

nature

20 July 1978

The two cultures – 1970's style

THE stereotype of the scientist—hair awry eyes flashing, a monomaniac oblivious to anything but his own research—which dates back at least to the 19th century, in Mary Shelley's *Dr Frankenstein*, is on the way back. The latest manifestation in the UK came with the demonstration in Trafalgar Square a few weeks ago against the Windscale reprocessing plant. The procession was headed by figures dressed in radioactive protection gear, clearly intended to be scary. Windscale certainly seems to have brought the image, always latent, out again; and last week Professor David Pearce (who contributes an opinion on the following page) put his finger on the reason. It is the lack of any indication that the nuclear industry, with which the public identifies the scientist, is prepared to discuss values, those human judgements about what is best by which most of us—including nuclear scientists—live our lives. Even further, the industry has at times appeared to be positively callous, with leading figures dismissing, in public and in private, fears of the proliferation of nuclear weapons as both inevitable and of little consequence.

In the 1950s, when western economics were growing rapidly, and new technologies appeared to be making life more bountiful, the average man in the western street was prepared to accept an apparently value-free scientist, above the ordinary run of life, as a mysterious but basically benign figure. He made magic but at least it was white magic. But now the other side, the unacceptable face, of technology has come uppermost, the magic appears to be black, and the scientist finds him- or herself seen yet again as hell-bent. Neither image is correct. But what is the root of this instability in the public's relation to the scientist? And what can be done to correct it?

Again we come back to human values, this time to their apparent absence in scientific activity. Science is almost by definition value-free: it's objective, 'public' knowledge that everyone can agree on. The scientists' goal, like the lawyers', is to reach value-free facts. An analysis of values, which must be open-ended as there are many possible value systems, is not his concern, so he becomes unskilled in such a task. The search for value-free knowledge ultimately impinges on human

values outside science, sometimes in very subtle ways, but most obviously through technology, which clearly changes people's lives. Technology is also open-ended, which leads to another problem as psychological studies of children have shown that those most likely to become scientists are 'convergent' thinkers—that is, most likely to concentrate on problems with a single solution. To deal with values and technologies a 'divergent' frame of mind would be more appropriate.

But how inevitable is this division? The distinction between the sciences and the arts in education is something that comes in roughly with the industrial revolution, and so may be a product of a particular society rather than something God-given. And there is one highly specific area where the scientist pre-eminently shows his or her sensitivity to human values—if only to eliminate them. This is at the scientific conference, where scientists go precisely to attach values to the results and theories they have read of in preprints and heard of on the grape vine, by assessing the individuals who are presenting them. At this stage valuations are often irrational, intuitive; but this is the mill through which science must pass before it takes on that purified, value-free look. To succeed in such valuations scientists must have a finely tuned awareness of their own kind.

Such skills are normally learnt on the job, so that the making of science, strangely enough for such an objective form of knowledge, is largely an unrecorded oral tradition (*pace* the attempts of sociologists and philosophers to record it). The education of a scientist, dealing in completed science, does not prepare him for the kind of judgements he will later have to make. In education, then, may lie the fault which leads to scientists unhappy at dealing with changes in values, making only a weak link with the world of which they are a part, and results ultimately in the precariousness of the scientists' image. The converger and diverger may not be personality types but roles into which people are driven. If so, it is a fault that sorely needs rectifying, if scientists are to make a sensible contribution—as they should—to the very broad debate on values and the shape of the future that our society is beginning to indulge in.



All this speculation, which you may take or leave, leads to one concrete recommendation, which you may judge on its own merits. This is that the political education of schoolchildren, which is a subject of much debate at the moment in the UK, would be brought to a fine focus and would serve the purposes outlined above, if it were to take as a project some technological

issue such as the need for fast breeder reactors, or the wisdom of expanding the Windscale reprocessing plant. Not only would this constitute a political education, but it would also draw together in the schools those two elements—the humanist and the scientist—that have been driven too far apart. Each would have a great deal to learn from the other. □

The nuclear debate is about values

David Pearce, Professor of Political Economy, University of Aberdeen and Director of the Windscale Assessment and Review Project, argues that any future nuclear inquiry must deal effectively with the differing values adopted by the nuclear industry and its objectors. The industry must drop its haughty assumption that opposition to it is 'irrational'.

DESPITE the acclaim with which Justice Parker's Report on the Windscale Inquiry was greeted in pro-nuclear circles, by Peter Shore and by many outside observers, it has had one effect which can only serve to strengthen and polarise the nuclear debate even more. It is easy to see that the selective, admonishing and often haughty style of the report was a tactical misfortune for the nuclear industry. For the industry all too often presents an image of infallibility, of being in possession of the truth, and of technological mastery in a context where such claims must defy credibility.

To deny this image of infinite credibility is to be branded as 'irrational', motivated by some immature ignorance, or reflecting some political stance unrelated to nuclear power—a kind of convenient 'coathanger' on which to hang an ideology not shared by the nuclear camp. It is difficult to know how far the industry reflects on the dilemma they create for themselves through this kind of argument. For the image of infinite credibility is non-sustainable: it is possible and credible to query the industry's claims. Yet to admit of error, doubt and to the unknown is to admit to risks in a wholly expensive and vastly important area of public investment. And such admissions may themselves carry risk of alarm and of generating further support for the opposition. It is a dilemma the industry must solve for itself. If anyone doubts that mistakes are made, they need only consult the recent work of David Henderson and Duncan Burn on the AGR programme (See P. D. Henderson, 'Two British Errors: Their Probable Size and Some Possible Lessons', *Oxford Economic Papers*, July 1977, and D. Burn, *Nuclear Power and the Energy Crisis*, Macmillan/Trade Policy Research Centre, London, 1978).

To be fair, many 'men of reason' characterise the pro-nuclear stance. They admit the risks and the unknown. They argue, legitimately perhaps, that the abandonment of nuclear power or even the failure to take on fast breeder options, entails an unacceptable sacrifice of material wealth since renewable energy cannot meet the demands of a growing economy, at least for the foreseeable future. Moreover, it is difficult to see why any sane mortal would perpetrate false claims so as to foist an unsafe industry on an unsuspecting public, unless, of course, the desire to be right and to toe the party line has overwhelmed that sanity.

A more sensible view is that those responsible for policy have made the value judgement that, given their belief that the risk is small compared to that embraced by other energy futures (witness the emerging environmental critique of the heavy use of coal), the benefits outweigh the costs. But if that is so, a strange feature can be observed. On the pro-nuclear side it is legitimate to argue that the values which

are assumed to prevail in current society, acquisitive, material values, are also the values of the future which will inherit a nuclear power programme. They may be right. The oddity is that, having embraced these values, there is an overt wish not to debate the equally legitimate value system which declares that a growth society is undesirable and therefore not in need of the nuclear infrastructure to sustain it.

In short, opposition to nuclear power can, and often does, derive from values which differ to those assumed or embraced by the industry and those who implement policy. If that is so, that opposition is rational even if it may be shown to be unshared by society expressing its views through some democratic process. What is needed is a clearer statement of what the costs and benefits of different energy futures look like, a need that places onus on the opposition as well as on the industry. To go further, we then need institutions to debate and evaluate those alternatives so as to ensure that ultimate democratic bodies are appraised of those differences. If the industry is seen to have been 'right' all along, so be it. What is disconcerting is its presumption, never proven, that what it wants is what the public wants. And proof involves more, not less, public participation; more concern for a credible image rather than hysterical outbursts about the irrationality and political motivation of the opposition. If much the same can be said for elements of the opposition, it merely demonstrates the low level to which the debate can fall.

We have elsewhere stated a more detailed rationale for bringing debates on the value system into the nuclear controversy (see *Windscale Assessment and Review Project, Interim Report*, June 1978). It is also clear that no such debate will resolve conflict, any more than the local motorway inquiry leaves the opposing parties much closer to agreement. But that observation makes it all the more important to be sure that the decisions are correct and efficient and are ones for which there is public accountability (who, for example, is accountable for Concorde, or the AGR programme?). There is no escape from the logic that, if the case for fast breeders or reprocessing plants or whatever, implicitly embraces values, then it is proper to afford legitimacy to an opposition which happens to embrace a different value system. This logic was evidently missed by Justice Parker as his report so clearly demonstrates when it discusses the alternative views he was presented with. That section of the report (see *The Windscale Inquiry: Report*, Vol. 1, 1978, Sections of Ch.8) is characterised by a perplexity and confusion which he was obviously happy to retreat from, to return to the technical issues which, science demands, must be provable one way or the other.

Failure to modify institutions to accommodate the values outlined here, and failure of the nuclear industry to change its image of self-righteousness are recipes for a different, and undesirable, nuclear controversy—one of conflict and not debate. To some at least, there is a very high cost to be attached to such a future. The inquiry over CFR 1, the first commercial fast reactor, will be one testing ground. Let us hope it occurs and is accompanied by a wider debate linked to better public information and proper political concern. □

US court rules against releasing research data

THE US Federal Court of Appeals has ruled that the raw data collected by university scientists in the course of federally-funded research programmes are not subject to the Freedom of Information Act, and need not therefore be disclosed to outside parties under the terms of the act.

This ruling was made last week on an FOIA application by a Boston-based medical group seeking to review the data gathered in a multi-university study of the treatment of diabetes mellitus, including the use of the oral hypoglycemic drugs. Largely based on the preliminary results of the study, which revealed that diabetics receiving the drug tolbutamide had a higher death-rate from cardio-vascular diseases than those receiving dietary or insulin treatment, the Food and Drug Administration recommended to physicians in 1970 that the use of oral hypoglycemics in the treatment of diabetes be carefully limited.

The appeals court's decision denies access to the raw data of the study by physicians contesting its conclusions. It therefore has implications for other areas in which the adequacy of data used for regulatory actions is challenged, and where the data has been collected by a non-government body.

Most applications for access to research data have so far come from public interest bodies such as the Washington-based Health Research Group, arguing that the public needs to be assured of the adequacy of safety and efficacy studies carried out on new drugs; one of the most controversial aspects of the proposed Drug Regulation Reform Act currently being debated by Congress, for example, is a move to make such information publicly available.

In contrast, however, the case on

which the appeals court ruled last week had been brought by a group of physicians concerned that deficiencies in test procedures may have led to unwarranted restrictions of drugs which they claim to have used both safely and effectively, a claim supported by many of their patients.

The tests in question were part of a \$7.3 million study initiated in 1959 by a group of thirteen separate universities known as the Universities Group Diabetes Program, and funded by the National Institute of Arthritis, Metabolism and Digestive Diseases. Over a period of nine to eleven years, data was collected on more than 1,000 adult diabetes patients receiving treatment at university-affiliated medical schools, and forwarded quarterly to a co-ordinating centre at the University of Maryland for statistical analysis.

Following the controversy that surrounded the publication of preliminary results of the survey—and in particular criticisms of the methodology—the FDA contracted an independent review of the study by the Biometrics Society which, although expressing some reservations, concluded that the UGDP's evidence for the harmfulness of tolbutamide was "moderately strong".

But the critics were still not satisfied. And in September 1975 a FOIA action was sought by a group of 178 doctors known as the Committee on the Care of the Diabetic demanding access to the raw data of the UGDP study, which was defined as consisting of the forms transmitted to the co-ordinating centre and the computer tapes and/or programmes on the basis of which the data were analysed.

The appeals court rejected the application—which had earlier been turned down by a lower court—by a two to one verdict. It ruled that the act,

which allows access to the records of federal agencies, should be interpreted literally, and that "only if a federal agency has created or obtained a record (or has a duty to obtain the record) in the course of doing its work, is there an agency record that can be demanded under FOIA."

The court said that research groups receiving federal grants do not thereby become government agencies, and that although an agency awarding a grant had a right of access to research data, it could not be compelled to exercise this right in order to generate agency records. The court also suggested that a decision that the UGDP raw data were agency records, and hence subject to the FOIA, would interfere with the autonomy of federal research grantees.

In a dissenting opinion, the third judge reviewing the case said that when taken together, three aspects of the research—that it was federally funded, that federal bodies had legal access to the data, and that the data was used for administrative decision-making—together implied that the materials were clearly agency records.

The applicants are seeking a rehearing, and if this is unsuccessful say they are prepared to refer the decision to the Supreme Court. And although the medical controversy over tolbutamide has calmed down in the past few years, further interest in the study has been generated by the conclusion, published in last week's *Journal of the American Medical Association*, that there is no evidence that the use of insulin or other substances designed to lower blood sugar in adults with mild diabetes will prevent progressive damage to blood vessels, the main cause of disabilities and death among diabetics.

David Dickson

House stands firm on Clinch River fast breeder

THE US House of Representatives has rejected a compromise formula offered by the Carter administration to end the long-standing dispute over the liquid metal fast breeder reactor project at Clinch River in Tennessee, making a presidential veto virtually inevitable.

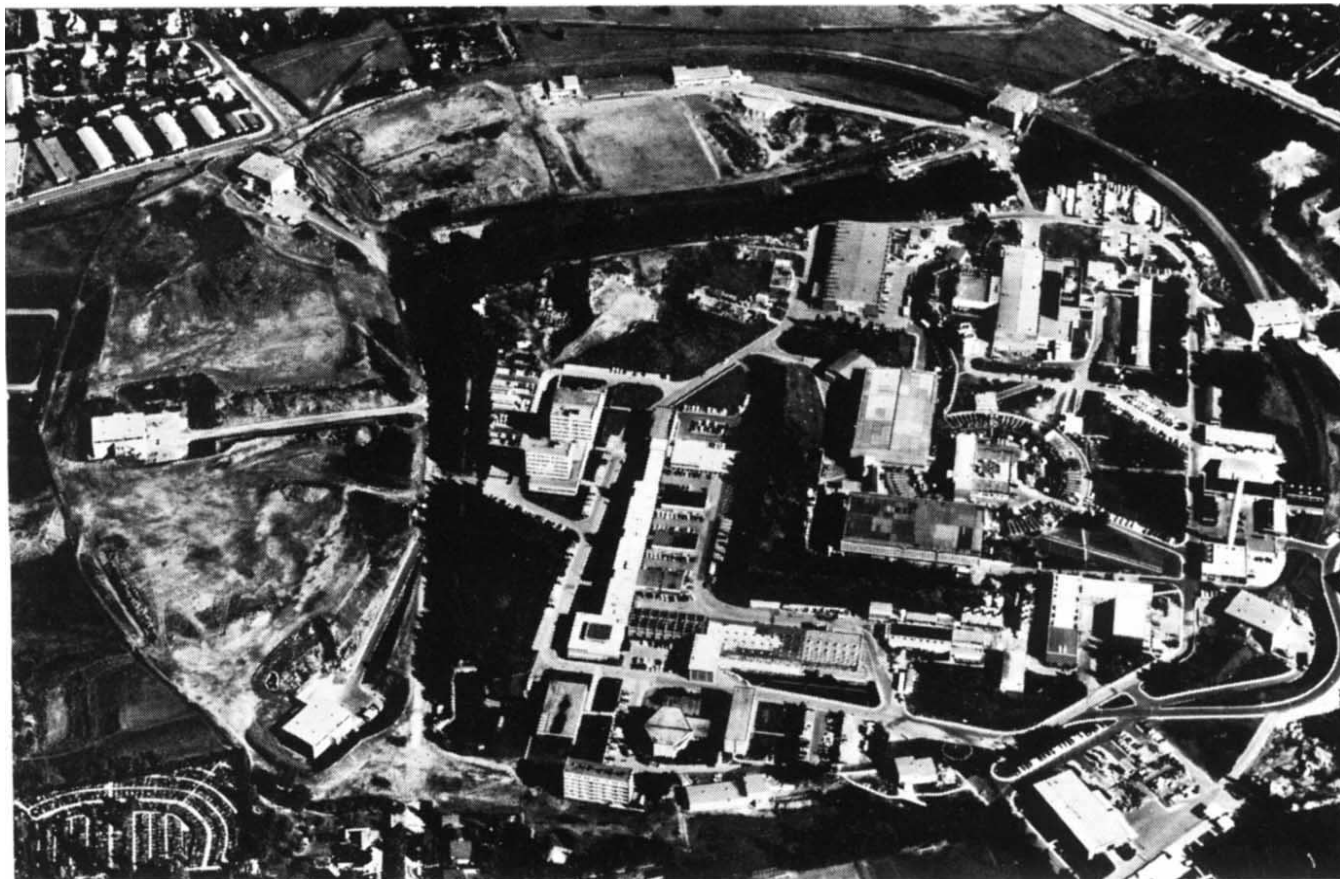
The House rejected last Friday an amendment to the Department of Energy's authorisation bill which would have allowed President Carter to kill the Clinch River project, on which \$800 million has already been spent, replacing it with a longer-term design study of a larger fast breeder using the uranium/plutonium and ura-

nium/thorium fuel cycles.

The amendment, which was defeated by 187 votes to 142, had been offered by Representative Walter Flowers (D-Ala.), chairman of the science and technology committee's subcommittee on fossil and nuclear energy research. Two months ago the same amendment was narrowly defeated by the science and technology committee, despite the support of the chairman Mr Olin Teague, and indications from the administration that congressional plans to proceed with Clinch River would almost certainly be vetoed. □



"Perhaps if he burns deutsch marks it would solve both his energy problems and our monetary problems!"



PETRA puts European physics in the lead

PETRA, Europe's 19 GeV on 19 GeV electron-positron storage ring under construction at the Deutsches Elektronen-Synchrotron laboratory (DESY) near Hamburg, has circulated its first beam around the ring and stored it "for several minutes".

At 10 pm on Saturday night, 15 July, a team of accelerator physicists headed by Dr Gustav-Adolf Voss successfully injected a beam of electrons along the axis into PETRA, and allowed it to circulate without any optical correcting magnets (sextupoles) switched on. Monitors detected no observable decay of the beam, but the beam was killed before a measurement of its lifetime could be made. This puts PETRA at least a year ahead of PEP, its American rival.

PETRA also won a race for funds within Europe against a British version of the same machine, EPIC, which was to have been built at the Rutherford Laboratory in Berkshire. It has been built with great speed and great efficiency by the German physicists, who seem to have adopted something of an American style to get the accelerator underway.

PETRA costs have been trimmed right down, and the machine has few frills—in contrast to its 10 GeV on 10 GeV predecessor, DORIS, which was arguably over-sophisticated.

DORIS ran into troubles in its commissioning, ending up with a much lower beam intensity—and hence data rate—than had been expected. Thus it was the American counterpart to DORIS, SPEAR at Stanford, California, that stole the limelight in the Nobel Prize winning discovery of charm, although DORIS ultimately made some extremely useful contributions.

With PETRA the boot seems to be on the other foot. If there is good physics to be discovered at these energies it seems likely that PETRA will discover it, rather than the American PEP, which will be left to play an important but supporting role. In conjunction with CERN's 400 GeV super proton synchrotron at Geneva, with its forthcoming high intensity lepton beams, Europe now seems set to take the lead from America in the high energy physics of leptons, which is where, in recent years, the most important discoveries of physics have come.

The next step at PETRA will be to make detailed measurements of the beam optics, and bring in the correcting sextupoles. The last obstacle to producing beam for experiments is likely to be the experiments themselves, the first of which is not likely to be ready before mid-September. This experiment

—PLUTO, a large magnetic detector working much like a big electronic bubble chamber surrounding a collision region—will begin to take data at around 10 GeV to establish an overlap with the previous machine, DORIS, and then go straight to the highest energies (38 GeV) to measure the total cross section for producing hadrons, divided by that for muon pairs. This quantity, R , made famous by experiments on charm, will tell the physicists how much interesting physics there is in between 10 and 38 GeV. The higher is R , the more physics—like new quarks and new leptons—there will be for PETRA to discover.

For the moment there will be a gap of a few weeks while DORIS, the old storage ring system which is involved in the injection of electrons into PETRA, is used for an experiment on its own: a search for the "upsilon prime" at a mass of over 10 GeV. The 9.5 GeV "upsilon", discovered by Leon Lederman last year and reconfirmed by DORIS recently, is believed to be a state consisting of a new quark ("bottom") and its antiquark (analogous to the J/ψ containing charmed and anticharmed quarks). The observation of the upsilon prime (an excited state of the upsilon) would help to confirm that interpretation.

Robert Walgate

Recombinant DNA hazards may be reassessed

A new basis on which to assess the probable hazards of recombinant DNA experiments and so determine the levels of containment at which they should be carried out is being considered by the UK Genetic Manipulation Advisory Group (GMAG). If accepted by both GMAG and the Secretary of State for Education and Science they would result in some experiments being downgraded, whereas others, now being carried out at minimum levels of containment, would need to be upgraded.

At present, containment levels both in the NIH and UK guidelines are broadly determined by the evolutionary relatedness of the cloned DNA to human DNA. Experiments involving bacterial DNA cloned in *E. coli*, unless it comes from pathogenic bacteria, may usually be carried out under minimum containment. Similar experiments with mammalian DNA require higher containment. Within each class of experiment the use of increasingly disabled hosts and vectors and increasingly pure DNA may allow lower levels of containment.

However, many inconsistencies in this blanket evolutionary approach have been pointed out. The new pro-

posals, mentioned at a recent meeting of the Biochemical Society in London, rely instead on an individual assessment of the probable hazard in each case. Although no details of the proposals have been announced it seems that they would have to take into account such factors as: the probability of survival of the host/vector system outside the laboratory; the probability of transfer of the recombinant plasmid to other bacteria; the probability of the DNA's containing a harmful gene; the probability that the genes carried on the recombinant plasmid could be expressed; and the probability that their expression would be harmful. The final probability of a harmful outcome would then determine the containment level needed.

Meanwhile the US National Institutes of Health revised guidelines are at present with Joseph Califano, the US Secretary of Health and Welfare, for review. And while they involve a number of exceptions and exemptions from the original NIH regulations, they are still based on a concept of evolutionary distance between the DNA involved and human DNA.

Eleanor Lawrence

US and China agree to expand science ties

THE People's Republic of China has set up a national commission with broad-ranging responsibilities for all aspects of science and technology policy, in line with the new regime's decision to make scientific and technological development one of the four main goals of government policy. It will be responsible for monitoring, co-ordinating and setting priorities and budgets for scientific and technological research. It will cover the activities of scientific academies, research institutions, and government ministries.

The head of the new commission, Mr Fang Yi, led a group of Chinese science administrators which held a series of meetings in Peking last week with an official government delegation of their US counterparts, headed by Dr Frank Press, director of the Office of Science and Technology Policy.

On his return to Washington, Dr Press said that the talks had been "serious, friendly, and provided a promising basis for future co-operation." The latter could include the exchange of data, advanced seminars, cooperative

research projects, "and a growing commercial relationship in the civilian, technical sector."

Dr Press said that the visit of the US delegation, which included the research heads of all the main government civilian agencies, was not meant as a gesture to the USSR, but was an attempt to establish the same type of relationship with China as the US currently enjoys with other countries.

During the visit, Mr Fang Yi told the US delegation that although there had been some increase in contact between scientists of the two countries since the signing of the Sino-Shanghai Communiqué of 1972, this was not enough. "New possibilities for Sino-US scientific and technological exchanges and co-operation need to be explored. We should make bigger strides and open wide avenues," he said.

Greater scientific and technological co-operation was "in the interests of both our sides and in accord with the desire of the people of the world to combat hegemonism," Mr Yi said.

David Dickson

Europe evaluates Seasat

EUROPEAN oceanographers and geodesists are collaborating with their American counterparts in assessing the usefulness of Seasat-A, the first purely oceanographic satellite launched by NASA at the end of last month. The European Space Agency is concerned at the possible cost of processing the enormous amount of data that the satellite will produce. For ESA, therefore, Seasat's evaluation phase is a time for assessing the satellite's value to European users before committing funds to building an expensive data processing facility.

So far two European experiments are involved in evaluating three of Seasat's four microwave experiments. They are JASIN, the joint air/sea interaction experiment; and a system of mobile doppler and tracking stations situated throughout Europe. Fourteen research vessels and aircraft are taking part in the former. They are making sea-surface measurements in the north Atlantic as a contribution to the International Decade of Ocean Exploration and their measurements can easily be compared with those taken by Seasat. The latter are assisting in the calibration of Seasat's instruments by determining the satellite's altitude to within 10 cms.

The fourth microwave instrument on board is a synthetic aperture radar—the first such instrument to go up in a satellite. It will provide all-weather high resolution (about 25 m) images of ocean waves, ice fields, icebergs and coastal conditions. As its data rate is very high (110 million bits per sec) it can only be operated in real time when data can be received by one of the tracking stations equipped to handle it (three are in the US, one is in Canada and one, owned by the European Space Agency, is at Oakhanger in the UK).

Seasat users in Europe are hoping to contribute to the evaluation phase of the synthetic aperture radar (SAR). They have already proposed seven possible experiments, of which they are sure that JASIN will be accepted—work on that will start soon.

The vast amount of data produced by the SAR makes its processing costly and time consuming. Computers at the Jet Propulsion Laboratory, where all the data is being processed, can only analyse 1 sec worth of data in 1 hr of computer time. If European users want to make use of SAR data during Seasat's application phase they will have to make provision for analysing the raw data and that could mean building a special data processing facility at an estimated cost of 1.5 mau.

Judy Redfearn

Putting science in its place

David Dickson talks to Joao Frank da Costa, secretary-general of the United Nations Conference on Science and Technology

A RECENT background document to next year's United Nations Conference on Science and Technology for Development, to take place in Vienna, opens with the blunt words: "The actual substance of science and technology will *not* be under discussion at UNCSTD."

The directness of the approach—as well as the sentiments behind it—has ruffled quite a few feathers in the international scientific community. However, it has left few in doubt of the views of the conference's secretary-general, Mr Joao Frank da Costa.

An economist and lawyer by training, with almost thirty years' experience in the Brazilian diplomatic service, Mr da Costa is no stranger to the United Nations scene, having frequently represented his country at sessions of the General Assembly. Nor is he a stranger to science policy issues. While serving at UNESCO he was secretary-general of the conference for the establishment of a Latin American Centre of Physics. More recently he has served twice as chairman of the UN Committee on Science and Technology for Development.

However, the scheme that he has helped to set up for UNCSTD reveals an approach that is significantly different from "science and development" type conferences of the past, which have tended to gather heterogeneous groups of experts to focus on particular issues of international concern.

Mr da Costa characterises this as a "top down" approach, producing impressive-looking plans of action, but all too often isolated from the mechanisms of social and economic change. In contrast, UNCSTD is being planned on a "bottom up" direction, confronting directly the complex social, economic and political entanglements of the real world.

Mr da Costa relates this to a new way of looking at science and its role in development, a role related to the philosophy of the New International Economic Order. "The traditional view of science as universally-available knowledge is no longer realistic, especially when the positive effects of science and technology are felt in some countries and not in others," he says. More realistic, but still with its limitations, is the approach which treats knowledge as a commodity and concentrates on attempts to facilitate the international flow of science and technology.

"The third view, and the one that

will be stressed at UNCSTD, is that science and technology are not really commodities, but are the product of social and political processes", Mr da Costa says. Related to this, he suggests the need for a new approach to science policy.

"The first attempts to develop science policies for the developing countries tended to produce abstract plans that floated in the air, not obeying any particular concept of development." Subsequently there was a shift to attempts to insert science directly into plans for economic development, with progress being measured essentially by economic parameters, such as the percentage of gross national product being devoted to research and development.

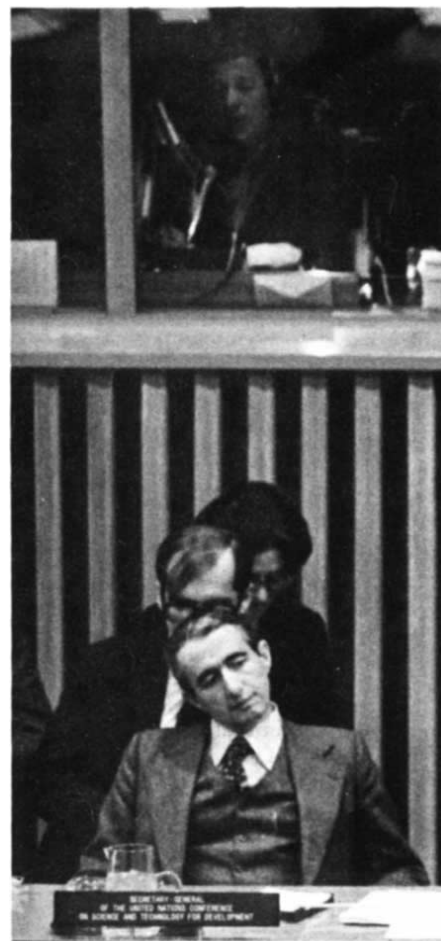
"The step which it is now necessary to take is to integrate science and technology into broad development objectives, not only concentrating on their economic aspects but on their social, political and cultural implications as well."

The agenda for UNCSTD reflects this approach, tackling problems in what Mr da Costa calls a "horizontal" manner with topics, each essentially social or economic in nature, grouped according to the way that they impede or promote the applications of science and technology to development problems.

Shifting the emphasis in this way has not been without its critics. Some have claimed that, as a result, the conference will not really be about science at all—or even about real-world problems—but fear that it could degenerate into a morass of bureaucratic haggling. Others fear that, by dealing only with government-sanctioned positions, the value of any outcome will be severely limited.

Mr da Costa expresses concern—although not necessarily agreement—with both these positions. For example, even within a strict development perspective, he claimed that there still exists a role for basic science in the developing countries "not necessarily for its own sake, but because it provides a good training for skilled manpower, and in this and other ways will reduce dependency on the developed countries."

As to political restrictions, he argues—in line with recent policies of the Brazilian government—that development cannot be left to market forces, but that "whether or not it is desirable, the state is nowadays the only entity



da Costa: translating science into a broader context

having the capacity and the necessary approach to take responsibility for the well-being of society."

In arguing that science and technology must always be seen as part of a broad social and political environment, Mr da Costa admits to sharing some of the concerns of those who, in recent years, have drawn attention to the costs, as well as the benefits, of science-based industrial progress.

"This popular reaction has been useful in developing a new perspective on science and technology, even though at times it may have been more emotional than informed. And whether the traditional western vision of science remains universally appropriate may well be an important topic of discussion at UNCSTD."

On one theme, Mr da Costa is in agreement with the critics of science, namely that scientists should no longer be treated as if they stood on pedestals, nor considered experts in development problems outside their own speciality. "The sight of a Nobel prizewinner signing a manifesto does not fool anyone any more," he says.

Where scientists should get involved in UNCSTD preparation, he suggests,

is in encouraging a three-way dialogue between governments, the public and themselves, and concentrating their efforts on the national papers. But as

he told a recent meeting of the American Association for the Advancement of Science, "scientists sometimes find it difficult to accept that the

actual application of knowledge to the problems of society depends on a decision-making process normally situated at the government level." □

Soviets climb down on mental hospital detentions

SOVIET misuse of psychiatry as a means of political repression may be decreasing, according to Mrs Sofiya Kallistratova, a retired Moscow lawyer. Mrs Kallistratova, who in 1970 had defended General Petr Grigorenko, one of the most famous of the 'psychiatric dissidents' said recently that the forced hospitalisation of dissidents as being mentally ill was now being phased out in the major cities and in cases when the dissident in question was well-known. This change of policy, she explained, was the result of the protest campaign which has been waged incessantly over the last few years.

Within the Soviet Union, such protest is headed by the dissident 'Working Commission for the Investigation of the Use of Psychiatry for Political Purposes', whose membership is said to include two psychiatrists who so far have remained anonymous. Since its foundation in autumn 1976, the 'Commission' has circulated a *samizdat* bulletin, listing cases of psychiatric repression; the latest issue (No. 9) of which also notes the arrest of Aleksandr Podrabinek, a member of the Commission.

Podrabinek is charged under Article 191—of the Soviet Constitution, with having maliciously circulated libellous fabrications harmful to the Soviet state. The main 'evidence' against him is his authorship of a 265-page *semizdat* document *Punitive Medicine*, dealing with political abuses of psychiatry. (Podrabinek was, until his arrest, a paramedic working with an emergency ambulance unit, a means of conveyance often used for the hospitalisation of dissidents).

Under Soviet law, proof that the allegations contained in the document are true or were published in good faith would constitute a complete defence. Since the history of recent trials under Article 191 suggests that such proof would not be permitted to be offered in court, a 'hearing' was called in London last week, to compile a dossier of defence evidence to be forwarded to the Soviet court and to be made available to relevant human rights organisations abroad.

The hearing, conducted by Mr Louis Blom-Cooper, Q.C. at the Institute of Advanced Legal Studies, called as 'witnesses' (either in person or via taped interviews) a number of pro-

minent ex-dissidents—Vladimir Bukovskii, who first drew world attention to the problem of psychiatric abuse, Dr Marina Volkhanskaya, who refused to administer neuroleptic drugs to political 'cases', Leonid Plyushch and General Grigorenko, former victims of the abuses.

A less familiar figure at the hearing was Dr Yurii Novikov, a psychiatrist, recently arrived in the West, who formerly worked at the Serbskii Institute of Forensic Psychiatry, a body which exercises overall control of all forensic psychiatry in the Soviet Union. Dr Novikov made no claims to heroism, stating, for example, that when

pressurised by a KGB official to certify a prisoner in a labour camp who was "making a nuisance of himself", he slid out of an awkward situation by suggesting that this was too complicated a case for him to decide, and recommended that the man be sent to the Serbskii Institute for a second opinion.

Perhaps because he makes no such claims for himself Dr Novikov's evidence is all the more credible. Certainly his 'inside' picture of Soviet forensic psychiatry is a frightening one. He asserts, for example, that one of the Assistant Ministers of Health, Evgenii S. Kuritsyn, who deals only with psychiatric matters, is a member of the KGB. He also gave a vivid account of the activity of Dr Georgii Morozov, a leading psychiatrist at the Serbskii Institute, to forestall criticism of Soviet psychiatry at the 1977 World Psychiatric Association Congress—notably by soliciting support from psychiatrists of the socialist bloc and from Finland.

Novikov's description of the Soviet pre-Honolulu campaign makes an ironic contrast to recent developments within the World Psychiatric Association. One of the results of Honolulu was the establishment of a committee to look into issues of professional ethics. Although the machinery for investigating such matters is still being worked out, there are suggestions that the committee might be set up in such a way that it is, by definition, totally ineffective. One informed source indicated to *Nature* that the WPA committee will not investigate any individual complaint of abuse; all such matters must be presented via the relevant national delegation. If implemented, such a situation would be worthy of Gilbert. A latterday Pooh-Bah would appear before the Committee as both appellant (on behalf of the individual making the complaint) and defendant (on behalf of his country's psychiatric profession). One can only hope that this is a misreading of the situation, and that the WPA will adopt an Amnesty-like situation, where national delegations present complaints of abuse relating to countries other than their own. In the meantime, the Royal College of Psychiatrists is to set up its own body to study such complaints wherever they occur.

Vera Rich



Podrabinek, now under arrest, and fellow dissident Irina Kaplun



Leonid Plyushch, speaking in Austria shortly after release from a Soviet mental hospital

Neurotoxins may go unrecognised

DAMAGE to the nervous system by industrial chemicals is still an under-researched subject, according to some scientists. This is probably due to old ideas concerning the mode of action of neurotoxins. Until a few years ago, it was assumed that peripheral neuropathy—often characterised by a tingling in the fingers and toes—was one of the earliest signs of nerve toxicity.

The 'dying-back' hypothesis, used to describe the phenomenon, held that toxic derangement of neuronal action began with the loss of metabolic activity at the nerve terminal, followed by progressive failure of the neuron in a retrograde (dying-back) fashion towards the cell body if impairment continues. In addition, it was generally believed that, if intoxication ceased, the nerve would regrow, and the subject would recover. And finally, the hypothesis held that it was the longest and largest diameter axons (supporting the largest metabolic load) which were the most vulnerable to neurotoxins.

These ideas are no longer tenable, according to Drs Herbert Schaumborg and Peter Spencer of the Neurotoxicity Unit of the Albert Einstein College of Medicine in New York. In a seminal paper, delivered at the recent New York Academy of Sciences conference on Public Control of Environmental Health Hazards, Schaumborg argued the case for a reappraisal of the neurotoxic hazard posed by industrial chemicals.

Two of those he and Spencer selected for study were acrylamide monomer—the precursor of acrylamide polymer—and a common industrial and laboratory solvent, n-hexane. Using cats as the test species, and the hindpaw as the limb of choice, the two demonstrated that acrylamide not only caused degeneration to regions of the axon prior to an effect on the nerve terminal, but that the most sensitive nerve in the hindlimb was one of 7–11 μm diameter, and not the 11–20 μm diameter axon.

With n-hexane, believed to be neurotoxic after conversion to the 2,5-hexamedione, degeneration in the hindlimb occurred in the nerves to the calf muscles prior to those near the foot. And in this case, it was the hindlimb which was attacked before the forelimb. So with n-hexane, Schaumborg believes that axon length and diameter are probably important in determining vulnerability.

In view of these findings, Schaumborg considers the term 'dying-back' to be inappropriate. Too many clinicians associate the expression solely with peripheral changes. Schaumborg and Spencer prefer to describe these pathological processes as 'central-peripheral

distal axonopathy'.

It is this use of the word 'central' which should cause the regulatory agencies to sit up and take note for it covers a striking and consistent observation made in the course of Schaumborg's studies with n-hexane. At the same time that changes were first noted in the vulnerable areas of the peripheral nervous system (PNS), axonal degeneration had occurred in the central nervous system (CNS). If exposure to the chemical is halted early enough, Schaumborg believes that the nerves may recover; however, if contact is prolonged, a stage is reached beyond which the CNS will not recover, the peripheral nerves being the only ones where regeneration is possible.

In cases of prolonged, low-level exposure to n-hexane, Schaumborg suggests, subtle changes may occur to the nervous system vital to memory and vision. And he adds that as neuronal function and number decline in the course of the normal aging process, individuals exposed to compounds such as n-hexane and acrylamide might experience premature deterioration in vision or mentation.

Schaumborg's investigations of a group of young Italian women exposed to n-hexane illustrate some of the problems in this field. The women, employed in 'cottage-industry' shoe factories near to Naples worked long hours in small, poorly-ventilated rooms. Rags saturated with a solution containing n-hexane were constantly handled in the daily work routine.

In one case, it was two years after inhalation of large amounts of n-hexane vapour that one of the women developed symptoms of cramping sensations in the hands, accompanied by weight loss and symptoms of anorexia. Two weeks after onset, the subject experienced cramp in her calves and developed an unsteady gait which was improved by the wearing of high-heeled boots. After a further two months, the legs weakened, and toes became numbed. She was finally admitted to hospital after a further two months, when walking to work became an impossibility. According to Schaumborg, two years later she still has a "slow, stiff-legged, waddling gait".

Some of the other women with prolonged high-level exposure to n-hexane have had a more devastating clinical history. Several have been quadriplegic for months; two have required transient respiratory assistance, two others have been left with visual impairment; and one adolescent has experienced a general decline in initiative and general intellectual ability.

The most extraordinary aspect of

these cases has been the assumption on the part of an Italian neurologist that the women were suffering from multiple sclerosis. The local general practitioner concerned with the cases refused to accept this prognosis, and Schaumborg was consulted. He pronounced hexane poisoning, and gave evidence to that effect during the course of a compensation suit filed on behalf of the women. The evidence was accepted, and they won their case.

Schaumborg considers that part of the blame for the misdiagnosis must rest with the shortage of research on neurological damage caused by chemical agents. Many of the regulatory agencies are not even aware of the problem, he says. And the one agency which is, the US Environmental Protection Agency, still persists in recommending an insensitive 'demyelination' assay for neurotoxicity studies. This assay, argues Schaumborg, will not detect the early primary axonal changes which eventually result in myelin breakdown.

One drawback encountered with the current tests for neurotoxicity is that, because they are bioassays, they are both time-consuming and costly. Behavioural tests are also employed; but, until the present time, neither type of test has been compared for accuracy. Now, however, a collaborative study, undertaken by the Albert Einstein Neurotoxicity Unit and the National Institute of Environmental Health, is considering simultaneous behavioural and morphological studies of the nervous system at various stages of intoxication in acrylamide-treated rats.

Although this collaboration will validate current screening processes for neurotoxic agents, investigators believe that future tests will be biochemical. Enzyme markers would be the best indicators and the enzymes of the Krebs cycle are currently under scrutiny. The Krebs cycle is viewed as being more promising than monitoring changes in essential fatty acid metabolism. Few neurotoxins—diphtheria is one example—attack the myelin sheath and involve fatty acid changes.

A more universal test is still a researcher's dream. For the moment, we do know that chemicals attack the nervous system in a number of subtle ways. Clinical tests will have to be flexible enough to detect a range of pathological symptoms. And it has to be remembered that both the PNS and the CNS are vulnerable to attack. In the case of the former, recovery generally occurs when intoxication ceases; but when the latter is involved, damage may be permanent.

Alastair Hay



Forest of dead trees at the centre of the explosion area. The huts in this 1931 photograph are those of explorer Kulik (right).

The 70-year-old mystery of Siberia's big bang

SEVENTY years ago this month, a Miss Katharine Stephen of Godmanchester, Huntingdon, wrote to *The Times*, commenting, in somewhat poetic terms, on the "strange light in the sky" which she and her sister had observed between midnight and 12.15 a.m. on July 1. "It would be interesting", she wrote, "if anyone could explain the cause of so unusual a sight".

Seventy years later, her question, and that of the many others who wrote to the press, remains unanswered. *The Times* did its best with suggestions of the *aurora borealis*, possibly associated with a large solar prominence currently under observation—or, alternately, debris from a Krakatoa-like but unrecorded volcanic eruption in some "little-known region of the world".

The source of the light did, indeed, lie in such a region—Siberia. But in those distant Tsarist times, the news which was relayed from St Petersburg to London was somewhat restricted. The few reports which appeared in local Siberian papers—in one case nearly two months after the event—never reached the notice of the St Petersburg correspondents.

A few rumours do seem to have trickled through and affected the fiction of the time—one pre-1914 short story (or rather "tall story") told of an explorer in Siberia who located a "lake" of solid diamond—produced by a meteorite which had hit a coal-seam. (The "lake" was, naturally, swallowed without trace by an earthquake before it could be exploited.)

Not until 1927, however, was any attempt made to locate the site of the Filimonovo meteorite when Leonid A. Kulik led an expedition to Siberia. (According to one unsubstantiated report, during the passage of the fireball a train had been halted at Filimonovo junction.)

The penetration of Siberia and its incorporation into the new Soviet economy was of great symbolic importance to the Russian leaders of the time—many of whom had spent long years as exiles there. The unexploited wealth of Siberia was proverbial—and the postulated giant meteorite might well have represented at least, psycho-

logically, an important economic asset.

What Kulik found, however, several days reindeer-journey from the Stony Tunguska river, was not a meteorite but a scene of weird devastation—radical fans of flattened trees with a small plantation standing without branches at the apparent epicentre. The "meteorite", if it existed, was never found.

The wrecked terrain presented a mystery, but was accepted without undue comment until 1945, when similar blast phenomena were observed at Hiroshima and Nagasaki. Suggestions soon began to appear that the disasters were similar in origin. It was recalled that the local population maintained that after the explosion, the reindeer had suffered from mysterious scabs. Tree-ring evidence suggested an enormous acceleration in growth rates since 1908—also characteristic of the nuclear-bombed cities. The "pillar of fire" which some natives recollected (20 years after the event) could well be called a mushroom cloud! Tunguska was to become, in the popular imagination, a nuclear explosion.

No one ever seems to have considered whether any of these phenomena are or could be characteristic of powerful explosions in general, rather than specific to nuclear devices. (Were the scabs on the reindeer, for example, simply burns from hot ash? What of the burgeoning of vegetation in conventionally-bombarded cities? Could an ultra-powerful but non-nuclear explosion produce a similar blast pattern?) The 'nuclear' hypothesis appealed to certain imaginations both in and outside the Soviet Union; particularly when this hypothesis was 'explained' by a postulated disaster to an extraterrestrial spacecraft! Moving from science-fiction towards science, the riddle of Tunguska attracted a wide range of explanations—a giant ball of ice and snow, which melted, ball lightning of unprecedented size, antimatter, a black hole. . . . By 1976, Igor Zotkin of the Committee of Meteorites of the Soviet Academy of Sciences could write: "I doubt if there is any recent discovery that has not been called on to explain the Tunguska

enigma".

The most recent expeditions have paid considerable attention to vitreous particles found in the peat bogs of the area and to the slight but definite increase in the radioactivity of the surviving trees in the area. As a result of their findings conventional Soviet opinion, as exemplified by Academician Georgii Petrov, Professor Nikolai Vasil'ev, and Vitalii Bronsten, now believes that the Tunguska explosion was caused by a small comet disintegrating in the atmosphere. (Certainly, the blast patterns suggest an aerial explosion). However, Professor Feliks Zigel of the Moscow Aviation Institute, drawing on eyewitness accounts (taken down some 15-20 years after the event), maintains that the body changed direction twice before impact and was hence a UFO.

And Alekesi Zolotov of Kalinin, described by Moscow radio in its anniversary programme as "another noted investigator" sticks firmly to his own explanation—a nuclear explosion of possibly, extraterrestrial, origin.

Zolotov is, incidentally, a Tunguska enigma in himself. His name turns up unfailingly in any discussion of the problem, and his theories, however bizarre to the scientific establishments, do at least get published. This in the Soviet academic system with its emphasis on procedure and its demand that each new piece of research be counter-signed by the head of department or institute is surprising in itself. Yet his own academic background seems obscure, and according to one physicist who worked for many years on the Tunguska site, Zolotov was originally simply an oil technologist, co-opted on to an expedition for his knowledge of the local terrain!

To celebrate the 70th anniversary of the "event" yet another expedition is to be launched this year. "Let us wait" said a Tass radio-commentary "for new facts to be elicited in the scientists' laboratories!" A laudable sentiment. But, if the expedition runs true to form, it may well produce yet a further crop of legends.

Vera Rich

correspondence

Careers for researchers

SIR,—We feel that your editorial (29 June, page 695) sympathised particularly with the problems of administrators picking their way through the 'minefield' of fixed term contracts and waiver clauses but gave short shrift to the scientists on short term research grants.

Short term grants may be acceptable at a junior level where postgraduate and postdoctoral workers need to move to gain experience but the virtual absence of tenured positions means that, for most, the only escape from the 'holding pattern' will be the dole queue. Consequently, research scientists feel that fixed term contracts will be regarded by the employers as a way out of not only their legal but also their moral obligations. How gratifying it would be to see administrators seeking ways of remedying the dearth of permanent positions rather than searching for loopholes in current legislation. Funds must be made available to develop a career structure for research scientists so that those of proven ability can concentrate on their research without the distractions of imminent redundancy.

The much overstated 'burning out' of scientific minds before retirement was alluded to in your editorial. If this is the reason for permanent positions not being available to research staff why then is it not also applicable to university teaching and administrative staff who, after all, are in the position of assessing the competence of those subject to short-term contracts. No one has questioned the vital role of senior scientists in, for instance, medical research yet neither the grant-giving bodies nor the universities are willing to accept the financial responsibility of establishing a career structure for them.

Research scientists, whose views have been unrepresented for so long, feel extreme anxiety over the implementation of these fixed term contracts and their continuing lack of career structure. We would welcome the views of others concerned about this problem.

J. J. AYRES M. W. RUSSELL
W. HARVEY H. A. SIMMONDS
M. KADLUBOWSKI A. UNGER

Guy's Hospital Medical School,
London, UK

SIR,—I was very disappointed to find in the editorial pages of *Nature* (29 June, page 695) a superficial and dismissive treatment of an urgent problem which affects large numbers of people involved in biomedical research.

Your view that the well tried system of employing young people on short-term contracts stimulates research by ensuring movement of people (and ideas) between laboratories, is only applicable where there is a natural progression from postdoctoral to tenured positions. It is generally acknowledged that the expansion of the universities in the sixties combined with recent cutbacks in money available for research, has resulted in the present critical situation where we have a plethora of well qualified, in some cases excellent, research workers in their late twenties

and early thirties who have no hope of gaining tenured employment. This situation can obviously be prevented in the future by discouraging young graduates from taking PhDs. However, it is quite a different matter to tell someone in their early thirties to find another career. Such a cavalier attitude represents a total abrogation of responsibility on the part of the establishment, and for those who are not convinced by such arguments, it is also a colossal waste of public money.

It should be remembered that the short term contract system serves two purposes. One is to discourage young scientists who are not suited for research and the second is to ensure that inordinate amounts of money and time are not wasted on unfruitful research projects. Thus the success of a (three year) project grant reflects the aptitude of the tenured grant holder as well as that of the postdoctoral employee. However, it is only the career of the postdoctoral worker which is in jeopardy if the project fails.

Restructuring the careers of non-clinical postdoctoral research workers, giving them an opportunity of security, will not impose constraints on research as your editorial suggests. On the contrary, under the present system there is no freedom to do research, and if this situation is allowed to continue there will one day be no scientists to do research.

MARLENE ROSE

Chester Beatty Research Institute,
London, UK

Morale at BARC

SIR,—We have read with great interest your report (6 April, page 489) on the proposed transfer of Dr R. Ramanna, Director, Bhabha Atomic Research Centre (BARC), employing nearly 11,000 workers including more than 3,000 scientists and engineers, as Director General of Defence Research and Adviser to the Defence Minister, Government of India. We completely agree with you that the policy of transferring active scientific workers to essentially administrative posts is normally undesirable. However, we feel that your remarks on Dr Ramanna's transfer do not accurately reflect the facts in two respects.

Firstly, it is not entirely correct to say that Dr Ramanna was an active scientist at BARC and would not remain so as Director General of Defence Research. The director of BARC coordinates the activities of some 40 divisions with the assistance of several directors of groups and lays down general policy related to various projects and organisational and budgetary matters. These are important and demanding duties and coupled with his other functions—mostly in public relations—they hardly leave time for active scientific work.

His transfer to the Ministry of Defence, where he would be formulating R&D policy and coordinating the work of several laboratories does not involve any essential change in the nature of his work. True, it can be argued that the projects to be coordinated and supervised by him in the Defence Ministry have little

relevance to his training as a nuclear physicist but this argument loses all of its force if it is remembered that at BARC Dr Ramanna has been coordinating projects in such diverse areas as chemical engineering, agriculture, food technology, reactor technology, lasers, vacuum technology, biology, nuclear medicines, chemistry and metallurgy.

Secondly, the impression given in your article, that Dr Ramanna's transfer was resented by the BARC scientists and other employees, does not reflect opinion at the centre. In fact, both the employees' union and the officers' association of BARC, which together represent all the 11,000 workers, issued strong statements welcoming the government decision to transfer Dr Ramanna from BARC.

There is absolutely no question of Dr Ramanna's transfer adversely affecting either the morale of the scientists at BARC or the quality of their work.

M. P. SANKARAN

BHABHA Atomic Research Centre,
Bombay, India

Parkinson's Law

SIR,—In a letter combining the literary merits of Sir Vivian Fuchs and William Shakespear (15 June, page 486), it is suggested that I failed to distinguish administrators and non-scientist staff who are not administrators in my analysis of the proportion of scientists in NERC research bodies (18 May, page 184). In fact, my argument is as follows: as a corollary of Parkinson's Law, the proportion of administrators in an organisation will increase with the size of that organisation, and therefore the proportion of non-scientists will increase.

The British Antarctic Survey, as Sir Vivian points out, is unique; but so is every other scientific organisation. When testing predictions about general laws we are interested in what these organisations have in common, not with differences amongst them. To ensure the objectivity of any comparison, it is necessary to ensure that the facts used are comparable in origin. This I did by deriving information about each organisation from exactly the same source, the NERC report for 1976–77. I was aware that the BAS runs ships, aircraft and field stations—but to have "corrected" the available data to allow for these would have been to treat the BAS differently from the other, equally unique, component bodies of NERC.

Nonetheless, if we accept Sir Vivian's plea that the BAS is a special case and exclude it from the analysis we get an equation very similar to the original:

$$A = 17.84 \log S - 1.31D - 2.26$$

where A is the percentage of non-scientists in an organisation, S the total staff and D the number of addresses. This regression accounts for 73% of the variation and again both coefficients are statistically significant. Parkinson's Law is validated irrespective of whether we consider the British Antarctic Survey or not.

R. Moss

Banchory, UK

news and views

What do the polyamines do?

from Seymour S. Cohen

MANY roles of the polyamines such as putrescine, spermidine and spermine in prokaryotic and eukaryotic cells are still undefined at the molecular level, but evidence has accumulated to suggest that one of their roles at least is to organise tRNA structure and activity. It has been known for more than a decade that tRNA can be converted from its inactive configuration to a more compact active form by the addition of monovalent cations or by relatively much lower concentrations of Mg^{2+} or polyamine. tRNA isolated from a variety of eukaryotic and prokaryotic cells has been found to contain one or two mols polyamine per mol tRNA (*Proc. natn. Acad. Sci. U.S.A.* **64**, 669; 1969; *Physiol. Plant.* **29**, 219; 1973). It was not surprising, therefore, that mixtures of Mg^{2+} with added spermine or spermidine facilitated ordered crystallisation of tRNA (*Nature* **220**, 1285; 1968; *Proc. natn. Acad. Sci. U.S.A.* **68**, 841; 1971).

Recent crystallographic studies have defined the positions of spermine and its binding analogue, ethidium, within molecules of yeast phenylalanine tRNA, confirming numerous earlier studies of the interactions in solution between tRNA and these organic

cations. The new physical studies have provided a more detailed insight into the requirements for these cations in organising tRNA structure and activity.

At low ionic strength, both spermidine and ethidium, which possess similar distributions of free and substituted amino groups, bind similarly to tight and less tight binding sites. Two tight binding sites available for spermidine in *E. coli* or yeast tRNA can also be occupied by ethidium in a fluorescent form. The substances compete for binding and can displace each other completely (*Nucleic Acids Res.* **2**, 1005; 1975). Numerous studies of cation effects on the reactivities of the thiouridine in *E. coli* tRNA indicated that one binding site for spermidine was in the vicinity of the relatively unstable stem of the dihydrouridine (D) arm. Both spermidine and Mg^{2+} appeared to act in this region sequentially and synergistically in stabilising and inducing folding of the tRNA (*Prog. Nucleic Acid Res. molec. Biol.* **17**, 15; 1976; *Biochemistry* **16**, 5729; 1977).

The enhancement of fluorescence of ethidium on binding to nucleic acid has been attributed (*Biochemistry* **16**, 3647; 1977) to a reduction in the rate of excited state proton transfer in a polar solvent. In a recent study of crystals of yeast tRNA^{Phe}, formed in the presence of Mg^{2+} and spermine,

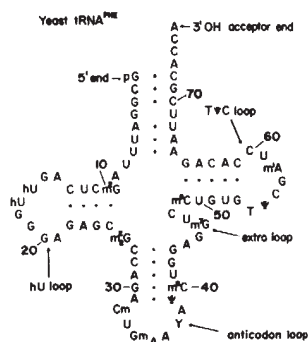
and washed and stained in ethidium, the fluorescent dye has been discovered (*Proc. natn. Acad. Sci. U.S.A.* **74**, 4821; 1977) to be bound specifically in a cavity in the 'P10' loop of the tertiary structure. The dye is not intercalated but is isolated from solvent in this pocket. The dye is stacked over U8 and is hydrogen-bonded to the phosphates P8 and P15 across the loop. The pyridinium nitrogen neutralises P14.

The structural positions of spermine and Mg^{2+} in the L-shaped structure detected in the orthorhombic crystals of yeast tRNA^{Phe} have recently been determined (Quigley, Teeter & Rich *Proc. natn. Acad. Sci. U.S.A.* **75**, 64; 1978). Two spermine molecules and four magnesium ions have been located in the structure. One spermine curls around P10 in a region in which nucleotides 8 and 9 join the D stem with the acceptor stem and P10 is close to P47 in the variable loop. Spermine then serves to separate the two chains. One NH_3^+ group of spermine is close to P9, and the two NH_2^+ groups are close to P47 and P46. The second NH_3^+ does not appear to be involved significantly in charge neutralisation and is possibly dispensable, as in crystallisation with spermidine which lacks an aminopropyl moiety.

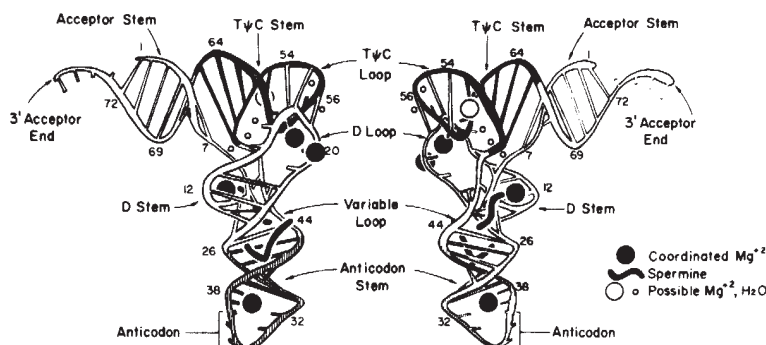
The spermine present in the crystalline structure remains for a finite time

Seymour S. Cohen is American Cancer Society Professor of Pharmacological Sciences at the State University of New York at Stony Brook.

a



b



in the complex dissolved from the crystals, and in that state prevents the insertion of fluorescent ethidium at its binding site (*Biochim. biophys. Acta* **447**, 110; 1976). Despite the apparent identity of this binding site for spermine and ethidium, comparison of the crystallographic data indicates that the polyamines and ethidium occupy overlapping but not identical sites and roles in this tight binding site close to P10.

It seems that one magnesium ion is also close to this spermine molecule, hydrogen bonding to P8, 9, 11 and 12, and serves to neutralise the charge associated in this bend of the structure. Two additional magnesium ions are located in the D loop coordinated to P19, 20 and 21, which exist at the intersection of the arms of the L-shaped structure.

A second spermine molecule is found in the deep groove of the tRNA structure extending from one end of the D stem into the anticodon stem. The cation exists in a hooked conformation and is thought to be hydrogen bonded to four phosphates on both sides of the groove of the helix. This distribution of neutralising charges appears to draw together the phosphates on the two sides of the groove, contributing significantly to the formation of a bend in this area. The fourth magnesium ion is in the anticodon loop, which is at the end of one arm of the L structure.

In early studies (*J. biol. Chem.* **246**, 4664; 1971) of the ability of an aminoacyl tRNA synthetase to transfer phenylalanine from the amino acid-adenylate complex to yeast tRNA^{Phe}, the transfer reaction was completely dependent on the addition of an oligovalent cation, such as Mg²⁺, spermidine or spermine. Conformational changes in the anticodon loop were detected by following the increase of fluorescence of the Y base (nucleotide 37) in this loop, which paralleled conversion of tRNA to an enzymatically active form.

Evidence for the cation-dependent activation of tRNA has been extended more recently in the study of a liver tRNA nucleotidyl transferase (*J. biol. Chem.* **251**, 6646; 1976) in which various Mg²⁺-free tRNAs deficient in terminal AMP were used as acceptors for this nucleotide. It was concluded that spermidine or spermine not only markedly stimulate the rate-limiting step in the catalytic reaction but also may be the normal counterions for tRNA *in vivo*. The multiple correlations of the activity of tRNA with the presence of the organic cations, the finding of polyamine in similar specific sites in tRNA freshly isolated from active organisms and in crystals and mother liquors of tRNA, and finally the recent structural work, help answer at least one aspect of the question 'But what do the polyamines do?' □

RNA polymerases in several respects.

Several primases are now known. The *E. coli* primase dnaG was the first to be identified by its requirement in the initiation of ϕ X174 and G4 synthesis. Replication in phages T4 and T7 requires phage-coded primases, the gene 4 product in T7 (C. Richardson, Harvard University; E. Lanka, Max-Planck-Institute, Berlin) and protein 41 and a newly-discovered 'protein X' in T4 (R. L. Burke, University of California, San Francisco). Priming in polyoma virus also seems to be directed by a primase (P. Reichard, Karolinska Institute). J.-I. Tomizawa (National Institutes of Health) has developed an *in vitro* system in which the ColE1 plasmid will initiate replication in the presence of RNA polymerase and DNA polymerase only. For initiation to start at the specific origin of replication however, RNase H is also needed.

The biochemistry of replication has been worked out in greatest detail for the small single-stranded DNA phages ϕ X174 and G4. These small phages depend almost entirely on *E. coli* replication proteins and have proved invaluable in unravelling the detailed biochemistry of initiation and priming. Their particular mode of replication enables discontinuous and continuous synthesis, which are inextricably linked in the replication of the bacterial chromosome to be studied separately. Complementary strand synthesis on the single-stranded (ss) viral template to produce the double-stranded replicating form (RF) is taken as a model of discontinuous replication. From A. Kornberg's laboratory, F. Meyer (Stanford University) described the mechanism of discontinuous replication in ϕ X174. They find that a pre-priming reaction is required. *E. coli* dnaB protein binds to ϕ X174 in the presence of DNA-binding protein and accessory replication proteins (n, n' and i) and provides a recognition site for the *E. coli* primase dnaG protein. dnaB acts as a 'mobile promoter', moving along the DNA and enabling the primase to initiate DNA synthesis repeatedly.

S. Wickner (National Institutes of Health) described the fine detail of the pre-priming reaction in ϕ X174. From the partial reactions of the replication proteins with each other she has constructed a scheme of ATP-driven reactions in which dnaB protein is transferred to the DNA from a complex with dnaC(D) protein in a reaction catalysed by replication factors X, Y and Z (corresponding to Kornberg's n, n' and i) resulting in a DNA complex with dnaB protein, DNA-binding protein, Y and Z, which supports primer synthesis by dnaG. Chain elongation requires similar formation of protein complexes on the DNA which provide recognition/binding sites for DNA

Replication: twenty-five years after

from Eleanor Lawrence

TWENTY-FIVE years after the first announcement of the double-helical structure of DNA, participants at this year's Cold Spring Harbor Symposium heard, as F. Stahl put it, how nature has solved the problems posed by the Watson-Crick helix.

The biochemical replication machinery has turned out to be extremely complex (for a recent review and a discussion of the possible reasons for such complexity see Alberts & Sternglanz *Nature* **269**, 655; 1977). Over the past decade, *in vitro* systems using purified constituents from bacterial extracts to support phage DNA replication have enabled phage and bacterial proteins essential for replication to be identified and their individual roles elucidated.

The development of similar *in vitro* systems described at the symposium for eukaryotic replication and for the animal viruses such as SV40 and adenovirus should soon provide a comparable eukaryote enzymology. R. M. Benbow (Johns Hopkin's University) described

an *in vitro* assay using fractionated soluble extracts of cytoplasm from unfertilised *Xenopus* eggs which has already been used to identify and purify several eukaryotic replication proteins.

Priming

Much of the biochemical complexity of replication seems to follow from constraints placed on the mechanism of replication by the properties of DNA polymerases.

The directionality of all known DNA polymerases, which extend DNA chains in the 5'→3' direction only, led to the idea that at the replication fork, synthesis on one strand must be 'discontinuous', with short chains continually being initiated and later joined to form a continuous new strand.

But because DNA polymerases can only add nucleotides onto a perfectly base-paired nucleotide, they cannot initiate DNA synthesis on single-stranded DNA. Synthesis must be 'primed', in many cases by short erasable RNA primers transcribed either by RNA polymerases or by primases, which differ from known

Eleanor Lawrence is News and Views editor of Nature.

polymerase.

An interesting contrast in initiation is provided by G4 which although closely related to ϕ X174 requires no pre-priming. One possible explanation for this difference comes from studies of the DNA sequences at the origin of replication of the complementary strand. Whereas ϕ X174 can initiate complementary strand synthesis at several sites, there is only one initiation site on G4. The primase alone recognises a DNA sequence corresponding to the *in vivo* origin of replication. This site consists of a 130-nucleotide stretch (N. Godson, Yale University) which is strongly conserved in the related phage ST-1 (which also needs no pre-priming) (D. Dressler, Harvard University) but not in ϕ X174. Dressler suggested that this relatively large sequence provides the nucleic acid counterpart of the dnaB protein. G4 starts RF $\rightarrow$ ss replication from a completely different origin which shows sequence similarity with that of ϕ X174 RF $\rightarrow$ ss origin in gene A. Synthesis is initiated here by nicking one of the strands and using the base-paired 3'OH end generated as a primer.

Evidence for discontinuous replication in bacteria was provided in 1964 by the discovery of 'Okazaki pieces'—small nascent DNA fragments of a few thousand nucleotides. However, recently Lehman and colleagues (see Olivera *et al.* *Fedn Proc.* **36**, 654; 1977) discovered that a part at least of the pool of small DNA fragments believed to be Okazaki pieces in bacteria can result from the misincorporation and subsequent excision of uracil into DNA. Subsequently a similar possible source of fragments has been found in cultured lymphocytes (M. Goulian, Rockefeller University) and polyoma virus replication (P. Reichard, Karolinska Institute, Stockholm). However, Okazaki pieces are too attractive a solution to the problem of directionality of polymerases to be lightly discarded, and were still an article of belief for most of the participants. In theory, true Okazaki pieces should be distinguishable by their RNA-primed ends but as B. M. Olivera (Stanford University) has found, some of the common criteria for identifying RNA primers do not distinguish between authentic RNA-primed fragments and pieces generated by uracil excision. Until it is possible to measure the contribution of RNA-primed fragments to the pool the extent of post-replication excision and repair remains uncertain.

However, replication of the double-stranded phages T4 and T7 provides a source of RNA-primed fragments for primer analysis. T. Okazaki (Nagoya University) described the analysis of the RNA primers on T7 'Okazaki

New transposons

from J. R. Saunders

It has been known for some years that genes specifying antibiotic resistance in bacteria are often found as components of translocatable genetic elements called transposons (see Cohen *Nature* **263**, 731; 1977). Recent work from several laboratories now suggests that functions other than drug and heavy metal ion resistance may be encoded on transposons. Recently it was reported (*Nature*, Jacoby *et al.* **274**, 179; 1978) that genes for the breakdown of xylenes and toluenes carried on TOL plasmids of *Pseudomonads* (see *News and Views* **269**, 470; 1977) also act as if they were part of a transposon. The *tol* genes are apparently borne on a translocatable element which is much larger at about 38 Mdal than any known drug resistance transposon. It will therefore be of some interest to determine the molecular structure of this transposon with particular reference to possible flanking repeat sequences. The ability of *tol* function to be transposed between replicons would explain both the heterogeneity observed amongst TOL plasmids and the deletion of *tol* genes which sometimes occurs when TOL⁺ strains of *Pseudomonas putida* are grown on benzoate (see *News and Views* **269**, 470; 1977).

The existence of a further transposon has been demonstrated by studying Lac⁺ plasmids. Such plasmids are of some importance clinically since they confer on host bacteria the ability to ferment lactose. This obscures a character of great significance in the routine identification of enteric pathogens such as *Salmonellae* which are normally lactose non-fermenting. A Lac⁺ plasmid isolated from *Yersinia enterocolitica* has been shown recently to contain a transposon carrying the *lac i*, *z* and *y* genes (Cornelis *et al.*, *Mol. gen. Genet.* **160**, 215; 1978). This *lac* operon is homologous with the lactose operon of *Escherichia coli* except that it probably lacks the *lac a* (galactoside transacetylase) gene. Unlike the *lac* operon of *E. coli* which is not apparently transposable, the lactose operon on this Lac⁺ plasmid is flanked by short (about 0.15 kilobase)

J. R. Saunders is a lecturer in the Department of Microbiology at the University of Liverpool.

inverted repeat sequences as might be expected of a transposon. These findings raise some fundamental questions as to the origin of the classical lactose operon. For instance, did the *lac* operon originate in *E. coli* and subsequently become incorporated into a transposon to assist its dissemination on plasmids to other enterobacteria? Alternatively, was the *lac* transposon acquired from some other organism early in the evolution of *E. coli*? Subsequent loss of transposability must then have been necessary to stabilise the Lac⁺ character. The answer to these questions may well become clearer when many more Lac⁺ plasmids have been investigated.

Plasmids specifying bacterial toxins are obviously important in determining the spread of pathogenic traits amongst the bacterial population. Of particular interest currently are enterotoxin (Ent) plasmids which confer on *E. coli* the ability to cause diarrhoeal disease. At the recent EMBO workshop on Plasmids in Berlin, Magdalene So (University of California, San Francisco) presented evidence which indicates that the gene determining the heat stable (ST) enterotoxin of *E. coli* is part of a transposon. This gene is flanked by inverted repeats of IS₁. However, showing transposition *per se* is not easy because there is no readily selectable genetic marker for enterotoxin production. This problem has now been overcome by artificially splicing a resistance marker into the middle of the ST gene carried by an Ent plasmid. A non-transposable tetracycline-resistance gene was inserted into an *EcoRI* restriction site in the ST gene. The subsequent transposition of tetracycline resistance from this engineered plasmid must therefore be due to the transposition of the toxin gene. This technique could obviously be applied more widely to show whether or not other bacterial genes lacking an easily recognisable phenotype are borne on transposons.

The discovery that a variety of genes carried on plasmids are transposable strengthens the view that bacterial replicons are assembled on a modular principle. Transposons are therefore turning out to be valuable pointers to the past, present and future evolution of bacteria.

pieces' formed *in vivo*. She finds that the majority of the RNA consists of AC-rich oligonucleotides ranging from dimers to pentaucleotides. The *in vitro* synthesised T7 primers found by

Richardson and Lanka seem to be mainly tetranucleotides with sequences ACCC and ACCA(C) respectively. No specific sequences seem to be present in the RNA primers isolated from

polyoma, SV40 and cultured lymphocytes.

The discovery that 'discontinuous' synthesis might be partly due to post-replication excision has also further confused the issue of whether *in vivo* even 'continuous' synthesis might be discontinuous. Even in ϕ X there are disagreements. Kornberg and his colleagues find that in their ϕ X system reconstituted from purified proteins, 'continuous' replication is indeed continuous, whereas in J. Hurwitz's laboratory 'continuous' replication of ϕ X in *E. coli* extracts turns out to be discontinuous, even when extracts from bacteria mutant in the uracil excision repair system are used. Kornberg suggested that the difference may be due to the use of *E. coli* extracts rather than purified proteins. The question of whether 'continuous' synthesis *in vivo* is to some extent discontinuous, remains.

Not all replicating DNA relies on RNA priming and 'Okazaki pieces' to solve the problems posed by the properties of DNA polymerase. tRNA primes the synthesis of DNA copies of tumour viruses, for example. One particularly ingenious solution is that of the linear single-stranded adeno-associated virus and parvoviruses, such as Kilham rat virus and human LuIII virus which have inverted repeats at each end. The hairpin duplexes formed by these repeats at each end of the molecule act as primers for initiation. Sequence studies in AAV (K. Bernes, University of Florida) have shown that the terminal repetition can occur in two orientations. This suggested a model for DNA replication in which DNA synthesis is initiated at the 3' hairpin and subsequent cleavage of the parental strand opposite the origin of replication regenerates a complete 3' end on the progeny. This leaves a base-paired 3' end at which DNA synthesis can be initiated to regenerate the hairpin sequences at the 3' end of the parental molecule.

Helix unwinding

One of the fundamental problems in both recombination and replication is unwinding the DNA double helix. Over the years a number of DNA-binding proteins, helix-destabilising proteins and untwisting enzymes have been found in prokaryotic and eukaryotic cells. The latest addition to the list is gyrase, first isolated from *E. coli*, which catalyses the introduction of negative supercoils into double-helical DNA in an ATP-dependent reaction. With a few exceptions (T4 for example) gyrase seems essential for *in vivo* replication in phage and bacteria. As described by M. Gellert (National Institutes of Health) gyrase consists of two subunits, A and B, coded respec-

tively by the *nalA* and *couI* genes, which explains the sensitivity of DNA replication to the antibiotics nalidixic acid and coumermycin (and novobiocin). In the absence of ATP, gyrase relaxes supercoiled DNA by a nicking-closing reaction. It also has ATPase activity. The combined nicking-closing and negative supercoiling activity of gyrase are presumed to act at some site at or beyond the replicating fork to relieve the positive supercoiling strain which builds up during replication and to aid unwinding.

The addition of oxolinic acid and subsequent treatment induces double-stranded cleavage of DNA by gyrase. Independent sequence studies described by Gellert and N. Cozzarelli (University of Chicago) of the presumed gyrase binding and nicking site revealed by this reaction indicate that gyrase recognises a sequence of some 11 base pairs and cuts a few bases to one site of it. Several such sites have been found in plasmid ColEI for example. J. C. Wang (Harvard University) described evidence that DNA is wound around the outside of the gyrase molecule.

The role of gyrase may not be confined to DNA replication. The effects of novobiocin and nalidixic acid on RNA synthesis suggest that gyrase or its individual subunits might be involved in opening up promoters for transcription. K. Mizuuchi (National Institutes of Health) has found an intriguing gyrase-mediated reaction. He has shown that *in vitro*, gyrase can induce palindromic DNA to 'snap out' of a circular molecule, producing a 'rabbit-eared' molecule from a plasmid containing inverted repeats. Interestingly however, when cells were transformed with these plasmids the only plasmids reisolated so far had lost the ability to 'snap out'.

Origins

Although the biochemical basis of replication is now well understood, studies on its control remain largely in the hands of the geneticists. A number of origins of replication have now been sequenced but sequences alone can give few clues to the mechanism of control. Primary structure homologies between *E. coli*, lambda and G4 origins for example may indicate important regions, and similar secondary structures can be constructed at several phage origins, even where the primary structures differ, offering possible recognition and binding sites for primases and polymerases.

The availability of nucleotide sequences however, now makes it possible to tie up identified base changes with mutant phenotype and altered affinity of the origin for known regulatory proteins. Such studies are now in

progress for a variety of replicons ranging from R plasmids to SV40.

An elegant study of the binding of T antigen to the SV40 origin (R. Tjian, Cold Spring Harbor Laboratory) has revealed a tandem array of three binding sites differing in their affinity for T antigen. Site specific mutations at restriction enzyme sites at the SV40 origin described by D. Shortle (Johns Hopkins University School of Medicine) should soon provide more detailed information on the interaction of the T antigen with the origin sequence.

Even in a replicon as well-characterised as lambda, however, the extent of the origin essential for initiation of replication is disputed. From genetic studies F. Blattner's group (University of Wisconsin) define one region, whereas G. Hobom (University of Freiburg) by assaying the ability of lambda origin fragments inserted into plasmids to drive plasmid replication finds that an extra region in *cII* is also required. Both groups however find that the *oop* region, once suggested as a source of the possible lambda primer is not needed. The specificity of the lambda O protein for the lambda origin has been traced to the terminal end of O which interacts with a specific nucleotide sequence within its own coding regions. (M. Furth, University of Wisconsin).

Indications that integrated SV40 virus replicates itself out of the host chromosome on excision were reported by M. Botchan (Cold Spring Harbor Laboratory). SV40 genomes integrated into non-permissive cells can be rescued by fusion with permissive monkey cells (which allow viral replication). This, together with the finding that the amount of DNA associated with the chromosome increases on fusion has led Botchan and his colleagues to suggest that SV40 replicates copies of itself out of the host chromosome by multiple rounds of bidirectional replication starting from the viral origin.

The derivation of the palindromic extrachromosomal macronuclear rDNA genes in *Tetrahymena* from the single integrated copy in the micronucleus present a similar type of problem. Yao and Gall have suggested that *in situ* replication could generate the first extrachromosomal copy of the palindrome, which consists of two copies of the gene inverted. However, no integrated copy of the gene can be detected in macronuclear DNA so that excision by some site-specific recombination mechanism with subsequent amplification is also an attractive possibility as M. C. Yao (Yale University) pointed out. □

* The XLIII Cold Spring Harbor Symposium on Quantitative Biology—'Replication and Recombination'—was held on 31 May–7 June, 1978.

Illegitimate recombination legitimised

from David Sherratt

TRANSPOSABLE genetic elements are unique segments of DNA able to insert at many different loci in DNA genomes (for a short review see Cohen *Nature* **263**, 731; 1976; for a treatise see *DNA Insertion Elements, Plasmids and Episomes* (eds Bukhari, Shapiro & Adhya) Cold Spring Harbor Laboratory, 1977). Although they have been best characterised in bacteria they were first detected genetically in maize by Barbara McClintock more than 20 years ago, and almost certainly exist in other eukaryotes.

The bacterial 'insertion sequences' (IS elements) were first recognised some 10 years ago when strongly polar mutations in the *gal* and *lac* operons of *E. coli* were shown to result from the insertion of specific DNA sequences (Starlinger & Saedler *Biochimie* **54**, 177; 1972). Most IS elements consist of 700–1,400 base pair sequences that can insert at numerous locations in the *E. coli* genome, often inactivating genes into which they insert and interfering with expression distal to the insertion. Several copies of these elements normally exist in the bacterial chromosome and plasmids such as F also contain similar sequences. Indeed the integration into the chromosome of F, to give Hfr's can utilise IS sequence homology between the two replicons.

More recently it has been shown that many plasmid-specified genes reside on transposable elements known as 'transposons'. Examples are genes for drug and heavy metal resistance, toxin production and novel substrate utilisation (see page 211). Transposons are generally larger than IS elements and sometimes have known IS sequences at their ends. Some transposons transpose at much higher frequencies than IS elements and clearly have genes that specify products necessary for normal transposition. The rapid evolution of new combinations of plasmid-borne multiple drug resistance is a consequence both of the ability of plasmids to be transmitted through bacterial populations by conjugation and transduction, and of the transposition of individual drug-resistance genes between plasmids having little or no homology.

Bacteriophage Mu, which seems to be able to integrate at practically any site in the *E. coli* chromosome is also a highly efficient 'transposer'—both of itself and of bacterial genes, and provides the only example of an autonomously replicating transposable element yet discovered.

As well as inactivating genes into which they insert, some IS sequences contain strong transcriptional 'stop' and 'start' signals by which they directly affect the transcription of neighbouring regions.

During two recent meetings, one on Plasmids in Berlin, sponsored by EMBO, and the other in Cold Spring Harbor on DNA Replication and Recombination, much new descriptive detail of these elements and their properties emerged, though little insight as to the mechanisms of transposition was gained.

Once inserted at any particular locus, transposons and IS sequences can mediate a range of *recA* independent 'illegitimate recombination' events requiring little or no DNA homology, which result in deletions and inversions of both host and element sequences as well as precise or 'nearly-precise' excisions of the element itself. For all these events the ends of the element are not only essential, but seem to be involved in the recombinational event itself. As N. Kleckner (Harvard University) described for the transposon Tn10 inserted into phage lambda, the 'inside ends' of the inverted IS-type sequences at the ends of the transposon seem particularly important.

Inevitably much of the new information reported at the meetings concerned the DNA sequences of the elements and the sequences at which they insert. Sequencing of a number of independent insertion sites for a given element reinforces earlier mapping data that showed that transposable elements have preferred regions, but not specific sites, for insertion. Surprisingly and most unpredictably a small host sequence adjacent to the point of insertion of all elements looked at so far is dupli-

cated, apparently during insertion, in such a way that the inserted element is bounded by a small direct repeat of non-element DNA. Insertion of IS1 generates 9-base-pair repeats (Calos, Johnsrud & Miller *Cell*, **13**, 411; 1978; Grindley *Cell* **13**, 419; 1978) as does Tn5 (H. Schaller, University of Heidelberg), Tn9 (M. Calos, Harvard University) and IS4 (P. Starlinger, University of Köln). Tn3 (E. Ohtsubo, State University of New York; S. N. Cohen, Stanford University) and the $\gamma\delta$ sequence in the F factor (N. Grindley, University of Pittsburgh) generate 5-base-pair repeats. Where a number of independent insertions have been sequenced it seems clear that insertion is not into any specific sequence. However, insertion cannot be completely random as there are known hotspots of some hundred nucleotides for several elements whereas others show regional specificity, preferentially inserting into regions some thousands of bases long as described for Tn1 by D. Botstein (Massachusetts Institute of Technology).

The significance of these duplications and the mechanism of their generation remains obscure, although two fairly straightforward possibilities present themselves. For example, if a nick is introduced on the 5' end of the sequence that is eventually duplicated, then replication and strand displacement of this sequence, followed by insertion of the element results in its being bounded by the direct repeat (on a single strand) (Fig. 1). Alternatively introduction of a staggered break about the sequence to be duplicated can give the above result on both strands (Fig. 2).

I can see no simple mechanism of transposition using known enzymology in *E. coli* that demands the observed duplication as an intrinsic part of either the insertion event or a subsequent retransposition from that site, though

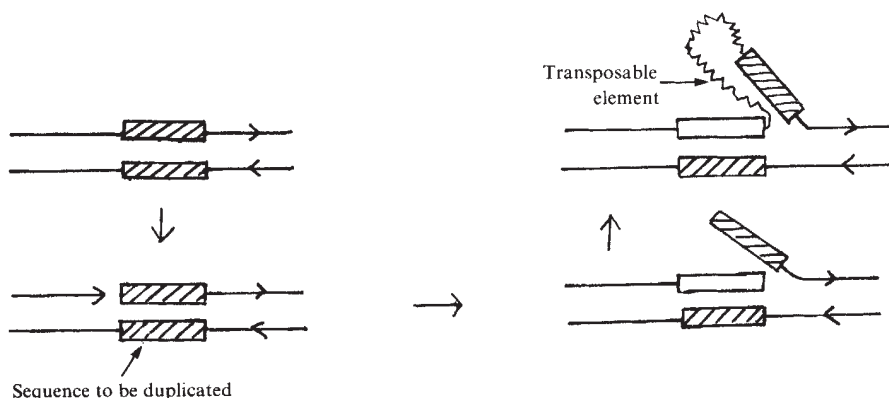


Fig. 1

David Sherratt is a lecturer in the School of Biological Sciences, University of Sussex.

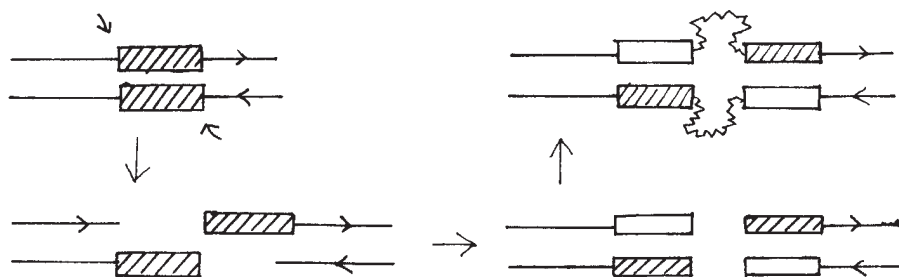


Fig. 2

interestingly other direct duplications result from transpositional events. When bacteriophage Mu is used to mediate transposition of a bacterial gene X, to a new replicon, X is finally found to be bounded by directly repeated Mu genomes. Similarly, experiments in Falkow's laboratory in Seattle and ours in Brighton, have shown that transposition of one class of mutant transposon from replicon A to replicon B, results in the acquisition by B of two directly repeated copies of the transposon sandwiching replicon A.

Sequence analysis of IS1 (N. Grindley; Ohtsubo & Ohtsubo *Proc. natn. Acad. Sci. U.S.A.* **75**, 615; 1978) and part of Tn3 (Cohen, Chou & Casadaban) shows a number of interesting features. IS1 has at its ends an inverted repeat of 30 bases while that of Tn3 is somewhat larger. Neither set of repeats is perfect and those of IS1 contain a number of promoter-like sequences as well as a sequence

homologous to that of the phage lambda attachment site. The promoter sequences are particularly interesting because of Ikeda and Kobayashi's report (*Proc. natn. Acad. Sci.* **74**, 3932; 1977) of RNA polymerase involvement in *recA* independent recombination in lambda. Analysis of the RNA specified by the ends of transposable elements could give some idea of the possible role of these sequences in transposition. Although transposable elements are not normally autonomous replicons (except those like Mu that also carry viral genes) and are only found integrated within other replicons, the Stanford workers have shown that if genes for autonomous replication are inserted into a transposon, then autonomously replicating transposons that are still capable of transposition can be isolated, albeit at a fairly low frequency. Whether this suggests that transposons normally have an autonomous (but non-replicating) existence remains an intriguing question. □

Hunting the W

from Peter Landshoff

It seems that there are in nature four different types of force between particles: the strong interaction that holds protons and neutrons together inside a nucleus, the electromagnetic interaction, the weak interaction responsible for nuclear beta decay, and the very much feebler gravitational interaction. Of these, the electromagnetic force is the best understood: it is described to extremely high accuracy by quantum electrodynamics. This theory quantises Maxwell's classical electromagnetic field, so that the field is given a particle-like character. The particles, photons, transmit the electromagnetic force between charged particles.

It has been believed for many years that there are also particles that transmit the weak force. These particles are

Peter Landshoff is Reader in Mathematical Physics at the University of Cambridge.

known as W particles. It is now hoped that their existence will soon be established. It has not yet been possible to produce them in laboratory experiments because, if they exist, they are very massive and so a very high energy accelerator is needed.

The mass m of a particle that transmits a force is related to the range R of the force by $R \approx \hbar/mc$. Thus, the photon has zero mass and consequently the electromagnetic force has infinite range, while the pion (discovered over 30 years ago) has mass $0.14 \text{ GeV}/c^2$ and gives the strong interaction a range of 10^{-15} m . (For comparison, the mass of the proton is $0.94 \text{ GeV}/c^2$.) Neutrino scattering experiments have shown that the weak interaction has very short range, such that the mass of the W particles is at least $20 \text{ GeV}/c^2$.

Salam and Weinberg have formulated an elegant quantum field theory which incorporates both the electromagnetic and the weak interactions. Their model is a first step in the unification of all four fundamental forces into a single relativistic quantum theory, which has long been an ambition of theoretical physicists. Predictions of the model agree well with experiment but the existence of W particles is crucial for its validity. In order that the Salam-Weinberg model be internally self-consistent, the mass of the W particles must be greater than $37.5 \text{ GeV}/c^2$, but a value close to twice this makes the model agree best with the details of the neutrino scattering data.

There are various plans, on both sides of the Atlantic, to construct accelerators of energy high enough to produce particles of such large mass. The Super Proton Synchrotron (SPS) in the European laboratory at CERN is particularly well suited to be adapted to provide the necessary energy. At present, the SPS accelerates protons to energy 400 GeV . They are extracted from the machine and are made to scatter on fixed targets. It is planned to inject into the SPS simultaneously both protons and antiprotons; these will circulate in the accelerator ring in opposite directions and will be made to collide head-on. Each particle in the head-on collisions will have energy 270 GeV ; according to special relativity the available energy in each collision then is equal to that of a $150,000 \text{ GeV}$ proton colliding with a fixed target.

At such high energy many interesting things are expected to occur as a result of the proton-antiproton interaction, among them the production of W particles. If the SPS collider performs as well as is hoped, there will be at least 10,000 interactions per second, and each interaction is expected to produce about 25 particles, mostly pions. There are indications from cosmic ray studies that in some interaction events the multiplicity will be considerably higher than this. In order to cope with the full complexity of the events, a sophisticated particle detector will be needed. A collaboration of more than 50 physicists, from Aachen, Annecy, Birmingham, California, CERN, Collège de France, Queen Mary College, Rutherford Laboratory and Saclay is proposing to build a huge detector, weighing 1,500 tons. As the SPS is underground the detector will have to be assembled 25 m below ground level.

It is estimated that one proton-antiproton interaction in every 10 million will produce a W particle. It may not be straightforward to recognise these events but, nevertheless, the race to find the W is on, and Europe hopes to win it. □

review article

High incidence area of cattle cancer with a possible interaction between an environmental carcinogen and a papilloma virus

W. F. H. Jarrett, P. E. McNeil, W. T. R. Grimshaw, I. E. Selman & W. I. M. McIntyre*

Cattle in upland areas of Scotland and northern England are substantially more prone to alimentary cancer than those of the immediately neighbouring lowlands, and epidemiological evidence implicates a combination of papilloma virus and bracken in the aetiology of the disease. Here Professor Jarrett outlines the circumstantial case against these agents and discusses its implications.

THE last two decades have seen a great expansion in the exploration of the molecular biology of tumour viruses with the development of new concepts and the unravelling of previously unsuspected cellular mechanisms. This rich harvest has not however, been paralleled by an increased understanding of the aetiology of the most commonly occurring malignancies, nor has it contributed to a higher cure rate. Burkitt's lymphoma and nasopharyngeal carcinoma in humans have been intensively studied because of their relation to Epstein-Barr virus and valuable insights have been gained. The greatest practical effects have been seen in vaccination against Marek's disease, a common malignancy of chickens induced by Herpesvirus. Techniques have been devised for the control of virus leukaemia in cats and cattle. It is in the common epithelial neoplasias that progress has been least. The aetiology of the major serious human tumours—carcinomas of the alimentary tract, breast and lung—remains obscure. The only virus-induced human epithelial tumour in which Koch's postulates have been met is the squamous papilloma or wart.

Suggestions that the aetiology of naturally occurring cancer may be multifactorial are not new but the large increase in knowledge of chemical carcinogens, hormones, viruses and genetic mechanisms has provided a set of tools for the dissection of candidate models for the induction of cancer. Here we describe a naturally occurring system which lends itself to this approach.

In the upland areas of Scotland and the north of England there is an extremely high incidence of alimentary cancer in beef cows. This cancer zone is accentuated by its juxtaposition to lowlands from which the particular tumours are almost totally absent. Sharp delineations of this kind are always interesting as the environmental factor which enhances the incidence may be identified by contrast and exclusion. Of further significance is an apparent interplay between a naturally occurring chemical carcinogen, possibly from bracken, and a papilloma virus.

We have made a detailed study of 80 cases and give an outline of the aetiological and epidemiological data as far as they are known; we also discuss the interest of these cattle as a general model system.

Alimentary neoplasms

Four types of neoplasm were found and their possible interrelationship is perhaps the most fascinating aspect of the data. The neoplasms were squamous carcinoma and papilloma of the

upper tract, and adenoma (or polyposis) and adenocarcinoma of the intestine.

Our interest was first raised by the finding of large numbers of upper alimentary squamous carcinomas and the 80 animals in this series were selected for the syndrome associated with this tumour. They were found at five specific sites: (1) the lateral dorsum of the tongue; (2) the soft palate and oropharynx; (3) the oesophagus; (4) the oesophageal groove and (5) a specific area of the rumen. Tumours were often found at more than one site in an animal and the organ most commonly affected was the oesophagus. Spread to other organs was found in 36%. Co-existent neoplasms of the intestine and of the urinary bladder were found in a large proportion of cases.

Papillomas, or warts, were found in the upper alimentary tract of 96% of the cows which had squamous carcinomas. The striking feature of these cases was the concordance of carcinoma sites and those of papilloma, the same five areas being involved. As will be seen later, these were typical of the alimentary squamous papillomas which we have found in an abattoir survey of 7,746 normal cattle in which the overall incidence was calculated as 19% (ref. 1). The papillomas in normal cattle were found in exactly the same sites as in the cancer cases. The striking difference between the two groups of cattle was in the number of papillomas present in an individual animal. In the survey of normal cattle which were examined at all sites, 95% of those with papillomas had tumours at one site, 5% had them at two sites and none had more; 77% had one papilloma and only 11% had more than three. In the cancer cases 76% had papillomas at multiple sites, 85% had more than five and 40% had more than 15; it was common to find more than 30 in a single case. It therefore seems that a marked amplification of the numbers of papillomas may precede the onset of carcinoma. Clinical examination of 366 cattle on ten farms in the cancer area revealed the presence of oral papillomas in 142 (39%). We have isolated a virus from alimentary papillomas and this is discussed later.

A number of cases showed incontrovertible evidence of the direct transformation of papillomas to carcinomas. All stages of this process have been studied and can be recognised macroscopically. Multiple transformed papillomas were present in individual animals. As tumours were found at various stages from benign to exclusively malignant, it is impossible to be sure that every carcinoma had arisen in a pre-existing papilloma and not by simultaneous transformation of previously normal epithelium. There was however, as we have mentioned, complete concordance of site of papillomas and carcinomas.

* University of Glasgow Veterinary School, Bearsden, Glasgow, UK.

It is not common practice in veterinary pathology laboratories to examine every inch of the bovine intestines in minute detail as they are very large and several hours of work may be involved. However, after the first 24 cases of upper alimentary cancer had been studied, we instituted a detailed examination of small and large intestines. The results were very surprising; 56% of the squamous cancer cases had neoplastic lesions of the intestine. Benign tumours occurred in three forms: an adenomatous polyp; a sessile plaque composed of similar hyperchromatic epithelium; and a rather more markedly proliferative lesion of the ampullae where the bile and pancreatic ducts open into the duodenum. These three lesions often co-existed and in about 50% of cases they were multiple, numbers in excess of 50 being not uncommon.

Adenocarcinomas of the small and large intestine were found in 9% of the cases studied in detail.

Of the cases with alimentary squamous carcinomas, 30% had bladder tumours. These consisted of haemangiomas (23%), fibromas (4%), transitional cell carcinomas (8%) and adenocarcinoma (1%), the total of percentages indicating that the simultaneous presence of more than one tumour type occurred occasionally. The haemangiomas were typical of the lesions of the disease known in many parts of the world as bovine chronic enzootic haematuria. The transitional cell carcinomas and adenocarcinomas were similar to those seen in man.

Incidence

Age-specific incidences of cattle diseases in Britain cannot be calculated because neither population denominators nor registered causes of deaths are available. We have carried out detailed necropsies on more than 5,000 cows from lowland farms and we have never found alimentary carcinomas and urinary bladder tumours of the types described. Many times this number of lowland cows have been examined clinically without evidence of the tumour-associated syndromes. Conversely, we have no difficulty in locating live cancer cases at any time in the upland area. Pirie² reported six bovine alimentary tumours from Scotland as 'unusual' cases of carcinoma. In a survey of 1.3 million cattle in Britain, only 3 out of 302 tumours found were alimentary; no urinary bladder tumours were recorded³. No particular breed was overrepresented in the present series, cases being found in all common breeds and crosses. The youngest cow with alimentary cancer was 7 years and the oldest was 18 years. Two-thirds of those of known age were 12 years or less.

The geographical distribution of cases is striking. More than 70% of the cases came from the west coast of Scotland and particularly from Argyllshire. The remaining animals had a widespread distribution over upland Scotland and the north of England. In 75% of cases the farms of origin were traced and without exception these contained marginal or hill land infested with bracken. Argyll with 100,000 acres of bracken has 15% of the Scottish total.

In a 2-year period we encountered 12 multiple-case herds; four were studied in detail and we were able to necropsy 30 animals which were being culled as uneconomic: 10 had alimentary carcinoma and 2 had urinary bladder tumours; 14 of the 18 which did not have carcinomas or bladder tumours had multiple alimentary papillomas.

Papilloma virus

The presence of virus in papillomas surrounding carcinomas was first suspected because of the discovery of intranuclear inclusion bodies characteristic of papilloma virus in a proportion of tumours. These occurred in localised cytological areas in each of which a high proportion of cells contained crystalloid arrays visible on electron microscopy. Subsequent study of papillomas from the abattoir survey showed that 13% had inclusion bodies while others had large pale vacuolated 'papilloma' cells. The nuclei of these latter contained varying numbers of virus particles. Virus was also visualised directly on electron microscopy by phosphotungstic acid negative staining

of suspensions of the tumour tissues. Purification was carried out by equilibrium centrifugation in CsCl. The particles had typical papilloma virus structure and could not be distinguished morphologically from bovine cutaneous papilloma virus (BCPV). The mean diameter was 55 nm and the density was 1.36 g cm^{-3} . Studies on the serology, nucleic acids, host range and cultural characteristics are in progress to establish the identity or otherwise of this virus with BCPV.

Transmission

When inoculated into the oesophagus, palate and skin, extracts containing virus produced typical papillomas.

The development of the skin papillomas was interesting in that the transformation process was biphasic in a fashion similar to that described by Olson⁴ for BCPV on skin inoculation. About 8 weeks after inoculation, tumours were palpable and biopsy showed that the main tissue mass of each was composed of fibrocytic cells derived from the upper layers of the dermis. Above this fibroma was a thickened epithelium with virus-containing cells in its upper layers. In the epithelium at each end of this swelling the formation of typical frond papilloma had begun.

In skin infected by BCPV the fibromas may regress after a month or two while true papillomas are more persistent. BCPV virus has a wider cell and host range than papilloma viruses of other species⁴; the developmental sequence in skin and oesophagus may be due simply to the force with which the original epithelial trauma is made and the depth of penetration of virus to subjacent tissues.

Aetiological implications

The main question raised by this study is whether the carcinomas in this high incidence area arose specifically from exposure of virus genome-containing cells to an environmental agent or whether the simultaneous occurrence of transforming papillomas and carcinomas of the alimentary tract was coincidental because of the exposure of both virus-transformed and normal epithelium to a primary 'carcinogen'. Current techniques of molecular biology should be able to resolve this problem as large quantities of purified virus can now be prepared. This means that tumours can be probed by nucleic acid hybridisation techniques for the presence of papilloma viruses in carcinomas of other epithelia such as the intestine and urinary bladder. In our epidemiological studies we have made a start at investigating the status of the two serious contenders for aetiological primacy or diarchy, bracken fern and papilloma virus. It should be underlined at the outset that the evidence that either is involved in the aetiology of alimentary carcinoma remains circumstantial and that up to the present, despite the apparent carcinogenicity of each in other species, there has been no experimental induction of bowel cancer in cattle by either.

Bracken fern has induced tumours in mice, rats, guinea pigs, quail and cattle⁵⁻⁷. Feeding fresh bracken resulted in tumours of the urinary bladders in cattle and guinea pigs. Dried bracken induced intestinal and urinary tumours in rats and lung adenomas and lymphoid neoplasia in mice. Milk from bracken-fed cows had been implicated in the induction of urinary bladder carcinoma in mice and rats. The exact nature of the bracken fern 'carcinogen' remains elusive and although various chemicals, such as shikimic acid, which have been extracted from bracken show some oncogenic activity, the major compound responsible has not yet been identified⁸. Bracken fern is eaten by humans in several parts of the world, particularly Japan but also in North America and New Zealand; preparations for human consumption have been shown to retain their carcinogenicity for rats. Certain areas in Japan have a high incidence of gastric carcinoma in humans and there is epidemiological evidence to suggest that eating bracken increases the risk of oesophageal cancer in man⁸. In cattle in Britain the most striking effect of eating large quantities of bracken is the disease known as acute bracken poisoning (ABP). The underlying cytological pathology

is radiomimetic and consists of thrombocytopaenia, agranulocytosis, aplastic anaemia and intestinal ulceration. One might postulate that the long-term effects of smaller dosage might also be radiomimetic and be associated with oncogenicity or immunosuppression; but the basic mechanism is as obscure as is that of irradiation. There was a history of antecedent attacks of ABP on many of the cancer farms in our series but there was high level of exposure to bracken in all. We have carried out a variety of bracken feeding trials in the last four years but we have not yet been successful in inducing cancer. One animal infected with papilloma virus and fed low levels of bracken over 4 yr was killed and proved to have 19 papillomas in its oesophagus.

Epidemiological studies have been carried out in two other regions where a high incidence of squamous carcinoma of the upper alimentary tract of cattle was found. The first of these was in Kenya. Plowright⁹ investigated a single valley, Nasampolai, where the expert Masai cattlemen were very familiar with a high incidence syndrome which proved to be due to squamous carcinoma of the rumen. Later, Plowright¹⁰ described 18 cases of which three also involved the oesophagus. The age incidence was lower than in Scotland and there was no obvious association with bracken or with enzootic haematuria although bracken was present and enzootic haematuria is well known in Kenya. Nine of the 18 had concurrent papillomas but attempts to show the presence of virus in cases from this valley were unsuccessful (Linsell, Orth, personal communications). The sharp physical localisation of the disease to one Kenyan valley among many which are superficially identical is fascinating. The disease did not occur in neighbouring valleys except when the cows had been pastured in Nasampolai earlier in life; they developed the disease within six months of translocation. Healthy cattle taken into the valley were said to acquire the disease in as short a time as one year. The overall annual loss from carcinoma was estimated at 2.5% of the total or 5% of adult animals which is not dissimilar to our experience in Scotland.

The other focus was in Brazil where Dobereiner¹¹ associated the syndrome both with the presence of bracken and with the co-existence of enzootic haematuria on the farms. Bracken clearing was said to abolish the disease. As in Scotland, the peak incidence of haematuria preceded that of carcinoma¹². Papillomas were found in the Brazilian cases but no virus was isolated.

To summarise, bracken contains a radiomimetic toxin(s) and a 'carcinogen(s)'; whether the major activities of each property

reside in one molecule or several is unknown. Two similar geographically restricted foci of cattle cancer have been described; in one, bracken has been putatively assigned an aetiological role but it has been denied in the other. One was associated with a high incidence of enzootic haematuria indicating the presence of bladder tumours, the other was not. In neither case was the simultaneous occurrence of intestinal tumours, such as we have found, recorded.

Turning to papillomas, these were recorded in Kenya and Brazil and Plowright⁹ suggested that they represented a stage in the development of cancer. We have shown that virus-induced alimentary papillomas are common in British cattle, being present in 19% of animals¹. These latter originated from farms with and without bracken fern. There was, however, a much larger number of papillomas per animal on the cancer farms and they frequently occurred at multiple sites. The situation is reminiscent of the exacerbations of multiple cutaneous warts in humans subjected to prolonged immunosuppressive therapy. Persistent papillomatosis has been reported in a bull with a deficiency of cell-mediated immunity¹³. There is also no question that bovine alimentary viral papillomas transform to malignancy¹⁴ and that this has been seen only in animals from the bracken areas.

Perhaps the main interest in this system is the chance to use the range of molecular biological technologies which have been elaborated in viral oncology over the last two decades to elucidate, in a naturally occurring tumour, the way in which an oncogenic virus, an environmental chemical and a host cell genome might interact to give rise to the phenotypic state of malignancy.

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articles

Vesicularity of basalt erupted at Reykjanes Ridge crest

Wendell A. Duffield

US Geological Survey, Menlo Park, California 94025

Average vesicularity of basalt drilled at three sites on the west flank of the Reykjanes Ridge increases with decreasing age. This change apparently records concomitant decrease in water depth at the ridge crest where the basalt was erupted and suggests substantial upward growth of the crest during the past 35 Myr.

UNLIKE the continuation of the mid-ocean rift system to the south, the Reykjanes Ridge has no axial valley, and water depth decreases steadily northwards until the ridge emerges near Reykjavik, Iceland. However, the transverse profile of the Reykjanes Ridge is very similar to that across the deeper rift system to the south, and such a shape is generally interpreted as the result of progressive crustal cooling and contraction with increasing age for a steady-state system¹. Accordingly,

oceanographers have²⁻⁴ concluded that the Reykjanes Ridge has been a steady-state, southwards-plunging feature throughout its existence.

New data from Leg 49 of the DSDP suggest a dynamic evolution of the Reykjanes Ridge, characterised by considerable upward growth of the ridge crest during approximately the past 35 Myr. As the ridge forms the south flank of Iceland, I suggest that such a history of ridge growth reflects the contemporaneous growth and eventual emergence of Iceland. This suggestion is consistent with the timing of the growth and emergence of Iceland proposed elsewhere^{2,5}.

New data

During DSDP Leg 49 three holes, at Sites 407, 408 and 409, were drilled on the west flank of the Reykjanes Ridge. These sites are aligned in a N60°W direction, virtually perpendicular to the ridge, along a flow line defined by the direction of spreading at the ridge crest (Fig. 1). The sites are positioned over seafloor magnetic anomalies 13 (Site 407), 6 (Site 408), and 2A (Site 409), corresponding to basaltic basement rocks that are estimated to be 35–36, 19–20 and 2.2–3.2 Myr-old, respectively⁶. These ages are consistent with ages of immediately overlying sediment as determined from fossils, with the possible exception of Site 407 where the first basalt drilled may be as much as 5 Myr younger than suggested by the seafloor magnetic anomaly⁷.

Basalt recovered at the three sites increases in average vesicularity from about 6% at Site 407 to 13% at Site 408 to 27% at Site 409. This marked increase presumably reflects a similarly time-related decrease in confining pressure induced by the column of water overlying the spreading ridge where the lavas were extruded and/or an increase in volatile content of the successively younger lavas at the time of their eruption. Moore⁸ has shown that vesicularity of seafloor basalt, when plotted against depth of eruption, defines a family of three curves, which are related to rock chemistry; at any given depth of eruption, relatively more vesicular rocks are richer in H₂O⁺, K₂O, P₂O₅, Cl and F. The least vesicular and thus most K₂O-poor group of lavas studied by Moore includes basalt from the crest of the Reykjanes Ridge and defines a parabolic vesicularity against depth of eruption curve (Fig. 2). The fact that relatively K₂O-rich basalt is more vesicular than its K₂O-poor counterparts is consistent with many field observations that indicate alkalic basalt is generally more volatile rich than tholeiitic basalt⁸.

Basalt recovered at Sites 407, 408, and 409 belongs to the most K₂O-poor group of basalt studied by Moore⁸, and the

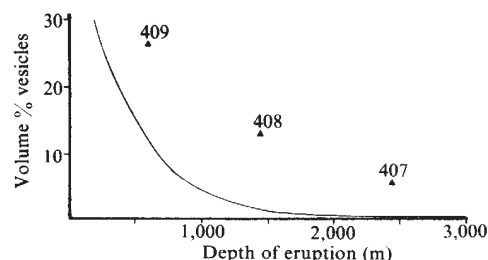


Fig. 2 Relationship between volume percentage vesicles in quenched glass margins of pillow lava and depth at which lava erupted for low-K tholeiite⁸. ▲, Possible depth for eruption of basalt from DSDP Sites 407, 408, and 409 as calculated in text. Vesicularity from Table 1. The symbols are higher than the curve of Moore⁸ because samples used to determine vesicularity are from relatively frothy interior of pillows, rather than quenched margins.

oldest lavas are most enriched in the constituents that show positive correlation with vesicularity⁹. Thus, the observed increase in vesicularity from older to younger lavas is contrary to the change expected from the effect of differences in rock chemistry and can confidently be ascribed to differences in confining pressure at the time of eruption. Theoretically, vesicularity of Leg 49 basalt may be compared with the data of Moore⁸ for a direct estimation of water depth at the time of eruption. Unfortunately, a less direct comparison is necessary, because of differences in samples from which vesicularity was determined.

Vesicles are typically distributed very unevenly in lava flows, so that interflow comparisons of vesicularity require careful sampling of comparable vesicle zones from different flows. Jones¹⁰ discussed this sampling problem in his study of vesicularity against depth of eruption for pillow lava extruded beneath snow and ice on Iceland; he measured vesicularity of samples from near the base of pillows for inter-pillow comparison. Moore⁸ attempted to minimise the sampling problem by using only the glass of quenched pillow margins. The sampling problem for the present study was aggravated because apart from glassy pieces, which are few and mostly used for rock chemistry, the original position of a sample from within a flow of the many penetrated at each site was unknown. Lacking such intraflow control, I studied the most vesicular portion of recovered core, reasoning that these samples would be most nearly representative of relative degree of vesicularity for intersite comparisons. Modal counts of vesicles were made on flat surfaces of split drill core as the core became available on board ship; the data are summarised in Table 1. A continuously recorded chart of drilling rate provides independent evidence that vesicularity determined by the modal counts is at least a semi-quantitative characterisation of average vesicularity at each site and thus that the indicated inter-site differences are not artefacts of sample selection.

During drilling of basalt at Sites 407, 408, and 409, the weight on the bit was maintained at 10,000 to 12,000 pounds and the bit was turned as closely as possible at 50 r.p.m. (D. Collins, personal communication). A new drill bit of the same type was used at each site and other drilling conditions were comparable, so that differences in resistance to penetration must have resulted primarily from differences in rock being drilled. Examination of penetration rate that was recorded continuously during drilling shows that the average time required to drill 1m of the basalt sequence was ~6, 5 and 3 min for Sites 407, 408, and 409, respectively. The only apparent physical difference among basalt from the three sites is an increase in vesicularity, 407 < 408 < 409, consistent with increasing ease of drilling. At Site 409 a Glomar Challenger record was established for total penetration through basalt by a single bit, which was probably possible because of the basalt's

Fig. 1 Index map showing location of DSDP Sites 407, 408, 409, and adjacent areas. One thousand fathom line outlines shape of Reykjanes Ridge.

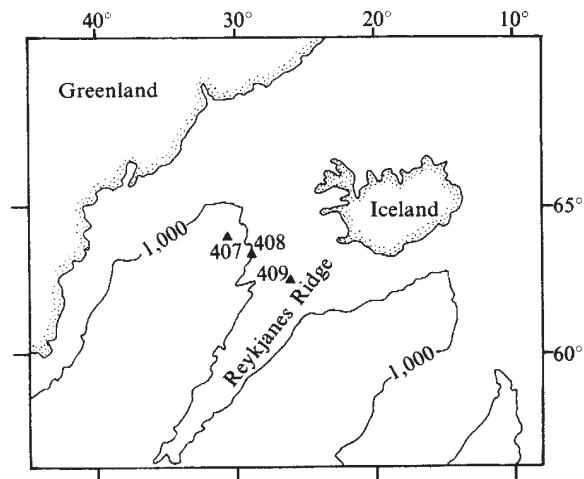


Table 1 Vesicularity, in volume percent, of basalt recovered at DSDP Sites 407, 408, and 409*

Site	Core, section, and piece	Vesicularity	Average
407	36-2-2A	5.9	5.6
	37-2-5	6.0	
	37-2-7	3.9	
	39-2-8	7.1	
408	36-4-3A	13.4	13.2
	37-2-8	11.2	
	37-2-14	13.6	
	38-2-9	14.6	
409	7-6-3	18.9	26.6
	9-1-6	38.1	
	9-2-10	31.3	
	9-3-5	13.7	
	12-1-9	20.3	
	13-1-4	22.8	
	13-3-6	32.6	
	14-1-1	37.7	
	14-1-5	33.1	
	15-2-15	28.6	
	16-1-6A	20.8	
	16-2-12	20.4	
	18-1-13	19.9	
	20-1-14	27.9	
	24-3-2A	19.1	
	25-2-7	27.6	
	31-1-15	34.9	
	31-2-7	30.4	

* Each determination represents a minimum of 1,000 counts on a grid fit to the size of available samples.

remarkably high vesicularity. Moreover, after early termination of drilling at Site 409, the bit showed very little wear, further evidence of the ease of penetrating such vesicular rock, and that vesicularity determined by the modal counts reflects real inter-site differences.

In addition, preliminary examination of benthic foraminifer assemblages from samples intercalated with or immediately overlying basalt suggests lower bathyal depths (1,000–2,000 m) at the time of deposition of the fossils at Sites 407 and 408, and upper bathyal depth (200–600 m) at Site 409; water depth at Sites 407 and 408 may have been closer to 1,000 m than 2,000 m, with that at Site 408 somewhat shallower than that at Site 407 (W. A. Berggren, personal communication). These conclusions are tentative and subject to refinement after further study of the foraminifer assemblages, but the preliminary suggestion indicates a history of decreasing water depth at the Reykjanes Ridge, similar to that indicated by contrasting vesicularity.

Calculated eruption depths

The depth of water in which basalt was erupted may be calculated for the two older sites assuming (1) that the Reykjanes Ridge has grown upwards to shallower water depths at a constant rate since 35.5 Myr ago, the approximate age of basalt drilled at Site 407; (2) that lithosphere generated along the ridge crest has subsided during this period in response to thermal contraction as described by Sclater *et al.*¹; and (3) that basalt of Site 409 was emplaced in about 600 m of water, the present depth of water at the crest adjacent to that site.

Basalt of Site 409 is now about 900 m below sea level, and the amount of subsidence expected from thermal contraction since eruption 2.7 Myr ago is ~440 m (Fig. 3, Table 2). Accordingly, the rate of uplift of the ridge is 52 m Myr⁻¹, (600 m + 440 m – 900 m) per 2.7 Myr. This rate may be used to calculate the water depth at which basalt of the older sites was erupted by the relationship $D_e = D_p + U - C$ where D_e = depth at eruption, D_p = present depth, U = uplift related to the

growth of the Reykjanes Ridge since eruption, and C = subsidence resulting from thermal contraction since eruption. The 52 m Myr⁻¹ rate of uplift corresponds to eruption depths of 2,446 m and 1,439 m for basalt of 407 and 408 (Table 2), respectively, reasonable figures when compared with present depths of 3,000 m along most of the mid-Atlantic Ridge to the south. The rate of uplift and the corresponding calculated depths of eruption for the older sites would be greater if basalt of Site 409 was assumed to have been erupted in water somewhat deeper than that at the present nearby ridge crest. However, the general result would remain the same. Calculated eruption depths and corresponding vesicularity at each site are plotted with Moore's⁸ data for comparison (Fig. 2).

Table 2 Depth of eruption (depth to crest of Reykjanes Ridge) based on assumptions in text

	Site 407	Site 408	Site 409
Age	35.5 Myr	19.5 Myr	2.7 Myr
Rate of ridge growth	52 m Myr ⁻¹	52 m Myr ⁻¹	52 m Myr ⁻¹
D_p	2,615 m	1,840 m	900 m
C	2,015 m	1,415 m	440 m
D_e	2,446 m	1,439 m	600 m

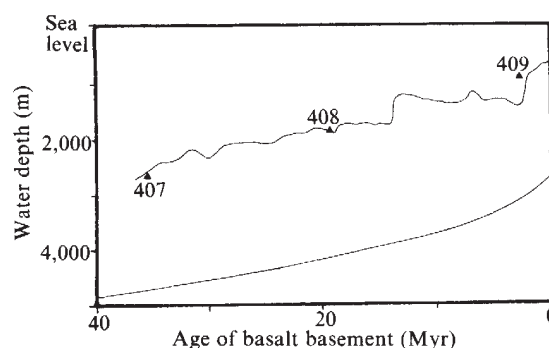
Discussion

How the Reykjanes Ridge might have become considerably shallower is speculative. Components of regional uplift and rapid accumulation of basalt, resulting from a relatively high eruption rate centred over a melting anomaly beneath Iceland, may be involved. Moreover, steep escarpments on the flank of the ridge (Fig. 3), parallel to the ridge crest, suggest some possible uplift along faults. These and other possible mechanisms of upward growth need further study.

The timing of upward growth of the ridge as suggested by contrasting vesicularity is also speculative. If, however, the growth of Iceland and the Reykjanes Ridge occurred contemporaneously, growth presumably began before 20 Myr ago, the approximate age of the oldest basalt exposed on Iceland^{11,12}.

Talwani and Eldholm² described the plate tectonic history of the North Atlantic and concluded that the formation of Iceland probably dates from ~27 Myr ago, the time of a westwards jump in the spreading centre, north of Iceland. This timing is consistent with the idea that the Reykjanes Ridge grew upwards at the same time as the formation of Iceland and that it began sometime between anomaly-13 time (Site 407) and

Fig. 3 Idealised age–depth curve for spreading sea floor¹, and seafloor topography along track of Glomar Challenger from crest of Reykjanes Ridge to Site 407, constructed from echo sounder records obtained during Leg 49. Positions of top of basaltic sections drilled at Sites 407, 408, and 409 (▲), with depth corrected for isostatic settling associated with overlying sediments. Age of basalt at each site is average for appropriate magnetic anomalies⁶. Sites 408 and 409 lie somewhat off the charted track.



anomaly-6 time (Site 408). Nilsen and Kerr⁵ concluded that the Iceland–Faeroe Ridge, part of a Tertiary land bridge between Greenland and Europe, subsided below sea level about 30 Myr ago, contemporaneous with initial formation of Iceland. This timing is also consistent with the idea that the Reykjanes Ridge and Iceland grew contemporaneously, beginning sometime between eruption of basalt drilled at Sites 407 and 408. If these two features did evolve together since ~30–27 Myr ago, ~7–10 Myr of submarine growth occurred, before Iceland emerged above sea level.

Received 16 April; accepted 2 May 1978.

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Offshore continuation of East Greenland dyke swarm and North Atlantic Ocean formation

Hans Christian Larsen

The Geological Survey of Greenland, Copenhagen K, Denmark

Offshore aeromagnetic data show that the coast-parallel dyke swarm of East Greenland continues on the continental shelf as a broad coast-parallel belt. A continental spreading centre at anomaly 24 has implications for the suggested origin of the North Atlantic.

IN their description of the coast-parallel dyke swarm of East Greenland, Wager and Deer¹ stressed the tectonic implications and a possible connection with the origin of the North Atlantic Ocean. They mapped a dyke swarm, approximately 10 km in width, over a distance of 360 km from latitude 66°15' to 69°15' N along the South-east Greenland coast. The offshore aeromagnetic data presented here show that this swarm continues on the continental shelf as a broad coast-parallel belt up to 80 km in width. Combining this with other data^{2–5} it is possible to delineate the swarm over a distance of 780 km between 63° and 69°45' N (Fig. 1), thus making it one of the most prominent post-Precambrian dyke swarms known. The dyke intrusion implies a considerable crustal dilation and it is postulated to constitute a continental spreading centre at the time of anomaly 24.

Intrusion

Within one part of the swarm exposed on land, the intrusive rocks constitute up to 99% of the surface^{9,10}. South of Kangerdlugssuaq (Fig. 1) the dyke swarm intrudes Archaean gneisses, whereas to the north early Tertiary basalts of the North Atlantic igneous province form the exposed¹ host rock.

According to Wager and Deer¹, the dyke swarm was intruded into the upper convex part of a major coast-parallel monoclinical fold which is responsible for the downwarping of the Denmark Strait area. This hypothesis has been questioned by Nielsen⁹ who views the monoclinical flexure as a result of block faulting and shearing in the westernmost part of the incipient North Atlantic Ocean. Two generations of dyke swarms exist in the Kangerdlugssuaq area: (1) a major pre-flexure generation of olivine tholeiites and (2) a less abundant generation of more varied composition, which is post flexure. More recent work¹⁰ has shown that on shore the dyke swarm forms an *en echelon* pattern.

As the dyke swarm postdates at least most of the lavas, the maximum age of dyke intrusion from palynological evidence⁷ can be put at 55–56 Myr. The Skaergaard intrusion cuts much

of the dyke swarm and fission track dating¹¹ on zircon from the intrusion gives an age of 54.6 ± 1.7 Myr, while the Kangerdlugssuaq alkaline intrusion clearly postdates the swarm and has given a radiometric age¹² of 50.4 ± 1.2 Myr. Thus, although no age determinations are available on the swarm itself an age between 56 and 50 Myr for the majority of dykes within the coastal part of the swarm can be concluded, and here, the major pre-flexure generation will be treated as a time-marker of approximately 55 Myr.

Palaeomagnetic data from the Tertiary of East Greenland are very limited. A reverse polarity is believed to predominate¹³ throughout the lava pile, as it does over most other parts of the North Atlantic lava province, and the lava extrusion are thought⁷ to have taken place just before anomaly 24. A few samples from the dyke swarm (T. F. D. Nielsen, personal communication) show normal polarity for the pre-flexure generation and mixed polarity for the latest generation. An unpublished aeromagnetic map of the Kangerdlugssuaq region (G. Goles, personal communication) shows a large positive anomaly related to the coastal exposure of the dyke swarm.

Restrictions of the dyke swarm

No-one has seriously questioned the restriction of the dyke swarm to the coastal area, although it has been argued^{2,5} using sparse marine magnetic data, that Tertiary dykes and basalts may occur on the shelf at latitude 63–64°N. The new aeromagnetic data shown in Fig. 2 have important bearings on that problem. These data define an impressive coast-parallel belt of high-amplitude-high-frequency anomalies. Definite separation of single anomalies is not possible which makes the polarity interpretation uncertain, but correlation by definition is drawn on positive polarity. Although a less perfect but still noticeable correlation can be obtained with mixed polarity (not shown), the anomaly belt is believed to reflect bodies with predominantly normal polarisation.

Power spectrum analysis within the anomaly belt defines a magnetic basement surface which coincides with the sea floor. The single anomaly members can be simulated approximately by vertical dyke models 800–2,000 m wide and continuing to a considerable depth. The apparent susceptibility amounts to about 5×10^{-3} (cgs units). This anomaly belt cuts directly across two orogenic boundaries (Fig. 1) and the major structures within these orogenic belts. The most reasonable geological interpretation of this highly magnetised basement is that of a coast-parallel dyke swarm. This interpretation is strongly

supported by the excellent correlation between aeromagnetic data and the southernmost exposure of the dyke swarm on shore (Fig. 2) and is an explanation of the large amounts of basaltic rock dredged on the shelf¹⁴. It is assumed that the offshore magnetic anomalies predominantly reflect the major pre-flexure generation of dykes.

Recent investigations¹⁰ in the coast zone have revealed very variable dyke density, with single dykes ranging from 1 to 20 m wide forming up to about 2 km broad high-density belts with more than 50% of intrusive rock. The offshore single anomalies are interpreted to reflect such density centres, that is minor swarms, and combined with the magnetic models, it is estimated that the intrusion of the major swarm implies a crustal dilation of the order of 50%. There is not enough seismic data to estimate the crustal change, but gravimetric data⁵ support the contention that the continental nature of the area was retained.

The western boundary of the major swarm, as delineated by aeromagnetic data (Fig. 2), forms an *en echelon* pattern, as also observed on shore¹⁰. A rapid easterly increase in depth to the magnetic basement defines the eastern surface boundary shown in Fig. 1. The continuation of the dyke swarm below the thick sedimentary section (2–6 km, H.C.L. in preparation) on the outer shelf is an open question, but a weak anomaly correlation in this area (Fig. 2) may indicate the presence of a considerable amount of the dyke swarm.

The aeromagnetic data presented here delineate the offshore continuation of the dyke swarm southwards from 66°15' to 64° (Fig. 2). Once the dyke swarm has been identified on the shelf, earlier data^{2–5} (not shown) including both onshore data and offshore aeromagnetic data from Project Magnet, allow the swarm to be mapped further north and south (Fig. 1) extending the swarm to 63° and 69°45'. How far the swarm extends still further to the south and north cannot be proved because of the lack of data, but according to the tectonic model presented here it cannot be very extensive.

The structure and width of the dyke swarm changes from south to north (Fig. 1). It forms a 10 km broad zone between 63° and 64°, giving rise to only one or two large anomalies (1,000–2,800 nT). The swarm broadens to approximately 60 km between 64° and 65° (Figs 1 and 2) and the number of anomalies rises to ~15 while decreasing in intensity (250–500 nT). A width of 80 km is attained just north of a flexure

affecting the swarm at 66° (Fig. 2). This flexure may reflect the landwards continuation of an oceanic fracture zone (Fig. 1) mapped by Johnson¹⁵ *et al.*

The dyke swarm juxtaposes north of 67° with the highly magnetised basement of the Iceland–Greenland ridge, and further north with the Thulean basalt province of central East Greenland (Fig. 1). Interpretation of magnetic data in these areas therefore becomes more complicated. The coast-parallel rim of high-amplitude-high-frequency anomalies between 67° and 69°45' as seen in the aeromagnetic data of Project Magnet, has been interpreted³ as reflecting the offshore continuation of the Tertiary lava pile exposed on shore. The frequency of large, correlatable positive anomalies in this area conflicts with this interpretation contrasting with the predominantly reverse magnetisation of the lavas, and it is suggested that the anomaly belt, at least in part, reflects the offshore continuation of the dyke swarm. If so the dyke swarm retains a width of ~50 km at 68° and gradually narrows northwards, constituting a zone only 10–15 km wide at 69°45'.

Regional interpretation

The longitudinal persistence and width of the swarm as now known precludes the intrusive mechanism proposed by Wager and Deer¹, but does not conflict with that proposed by Nielsen⁹. The age of the swarm and the considerable dilation it suggests, makes it obvious that it should be interpreted in terms of North Atlantic Ocean formation. Anomaly 24 is the oldest identified anomaly in the North-east Atlantic²⁷ north of 56°, but does not constitute a continuous feature. It terminates outside the Rockall Plateau^{16,18,27} and the South-east Greenland shelf^{17,18} well before the gross termination of younger seafloor spreading anomalies against the Faeroe–Iceland–Greenland Ridge, and according to Talwani and Eldholm¹⁸ (and Eldholm, personal communication) anomaly 24 is first easily identified to the north of the Jan Mayen Fracture Zone. Undated oceanic crust of pre-anomaly 24-age has been found^{18,28,32} to occur marginal in the North-east Atlantic and the Arctic Ocean.

The dyke swarm is largely restricted to the area defined by lack of anomaly 24 and, in fact, forms an *en echelon* connection between the northern and southern parts of anomaly 24 when plotted in a pre-drift situation¹⁹ (Fig. 3). Interpretation of the

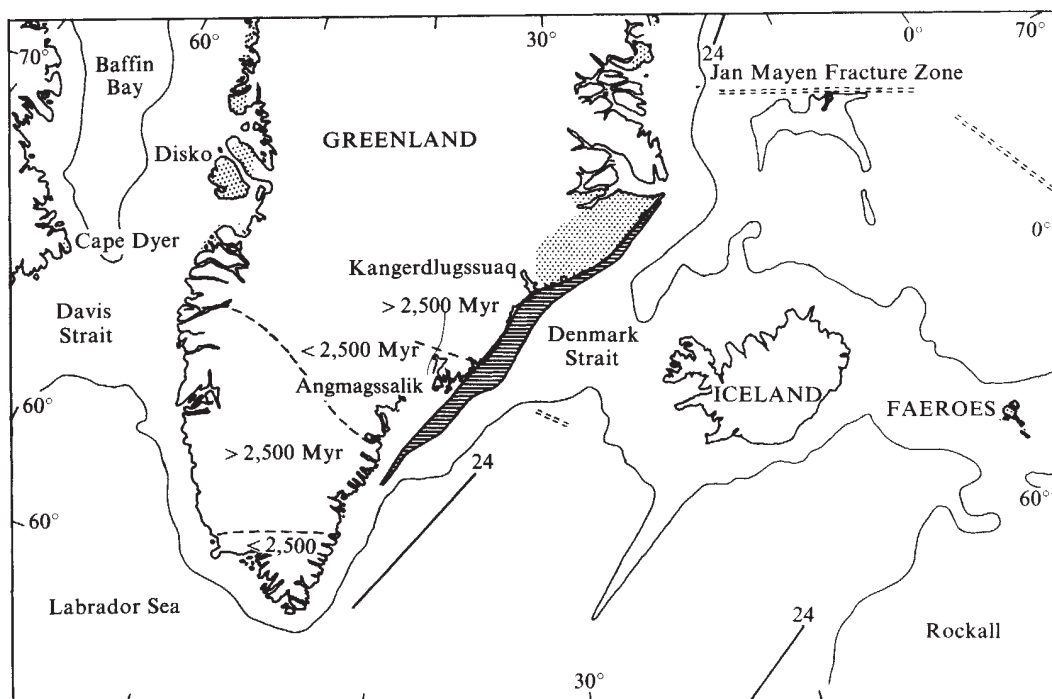


Fig. 1 Horizontal ruling marks the distribution of the East Greenland coast-parallel dyke swarm as deduced from the new aeromagnetic data (see text) and previously published data^{1–5}. Dots mark Early Tertiary lava plateaus within the North Atlantic Igneous Province. Selected fracture zones are shown by double broken line. Position of anomaly 24 from Talwani and Eldholm¹⁸, unnamed fracture zone from Johnson *et al.*¹⁵. Polar stereographic projection with 500 fathom contours are shown.

East Greenland coast-parallel dyke swarm as a continental spreading centre at anomaly-24 time, therefore, seems reasonable, and agrees with the available palaeomagnetic data. The proposed interpretation suffers from different uncertainties due to incomplete palaeomagnetic data and lack of direct connection between the dyke swarm and dated oceanic crust. A post-anomaly 24-age of the dyke swarm cannot for several

Fig. 2 Total magnetic intensity along flight tracks marking a level of 54,000 nT. The survey was flown at an approximate altitude of 250 m. Good anomaly correlation are shown as dashed lines, tenuous ones as dotted lines. Crosses mark the area with southernmost exposures of the main dyke swarm according to Wager and Deer¹. The area linking the onshore dyke exposures and the offshore data forms an *en echelon* pattern in close agreement with the most recent onshore data (ref. 10 and J. Myers, personal communication). Mercator projection.

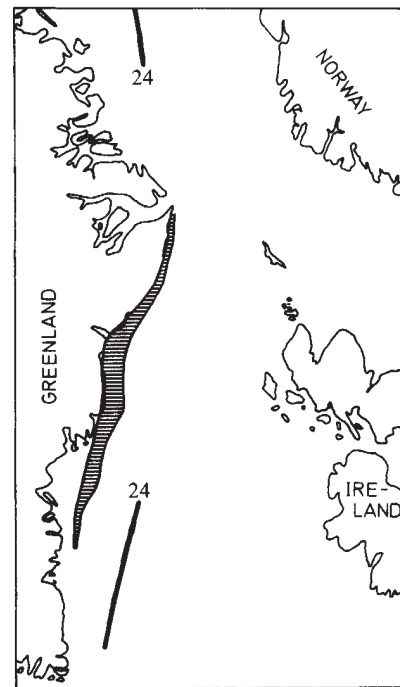
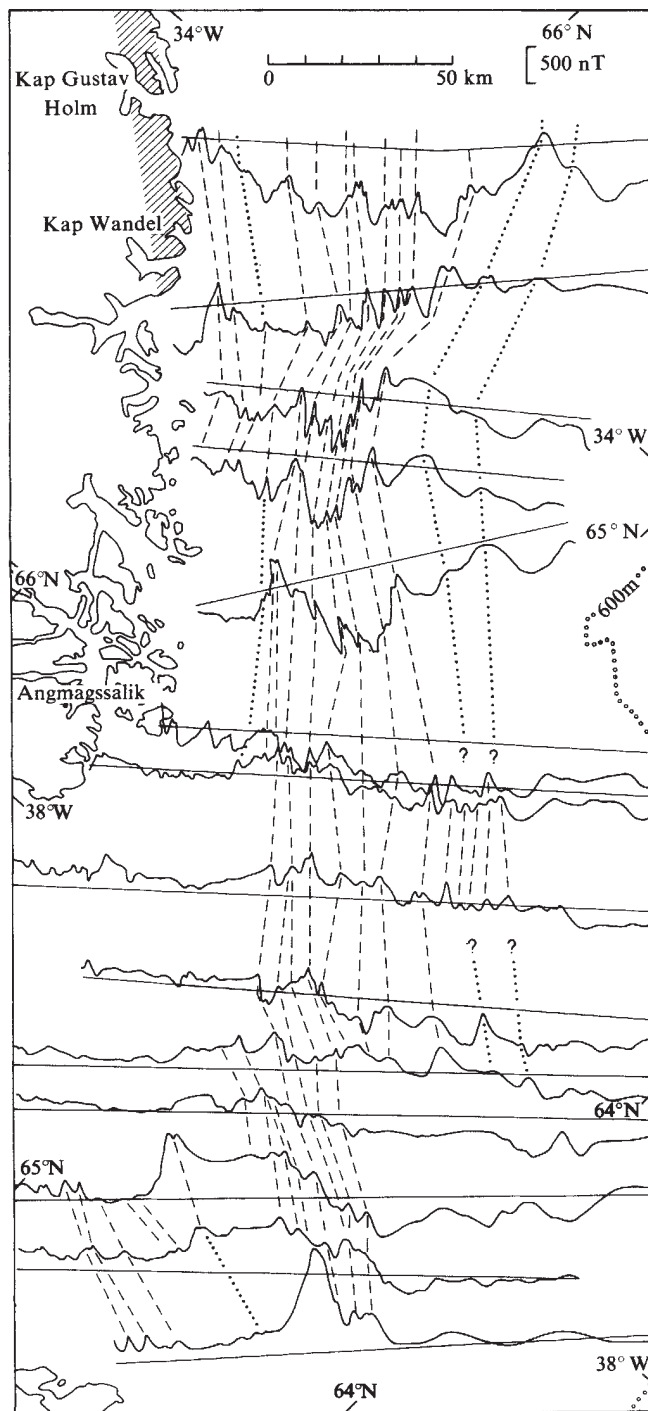


Fig. 3 The East Greenland coast-parallel dyke swarm plotted in the pre-drift configuration of Le Pichon *et al.*¹⁹. Position of anomaly 24 from Talwani and Eldholm¹⁸ and fixed relative to Greenland. Polar stereographic projection.

reasons be accepted, whereas a pre-anomaly 24-age may be a possibility that agrees with the above mentioned observations of a very early drift phase.

The area defined by continental crust extension in the early drift phase contains three megatectonic features which probably relate to the type of crustal dilation: (1) the North Atlantic Igneous Province is largely restricted to this area; (2) the Faeroe-Iceland-Greenland Ridge falls within this area; and (3) the spreading history of the area is more complicated compared to the simple and symmetrical spreading pattern to the north and south¹⁸.

The composition of basalts from the Faeroe-Iceland-Greenland zone contrasts with normal ocean floor basalts, the former being enriched in K, Ti, other incompatible elements and iron (FETI basalts). According to O'Hara²⁰ the difference is a consequence of differentiation in the thicker crust which the FETI basalts have penetrated. Other workers²¹ invoke an upwelling of hot, deep-seated mantle material ('hot spot') with a characteristically undepleted composition. According to Vogt¹⁶ and Schilling²¹ this hypothetical hot spot activity constitutes the driving force responsible for the Tertiary rifting and drift in the North Atlantic area.

Conclusions and wider relationships

Although the data presented here has no direct bearing on the basalt genesis, the early spreading history outlined stresses the importance of the tectonic setting of the basalt province, and for the following reasons, I suggest that the lavas and the dyke swarm originate from the same magma source: (1) the magma volume involved in the extrusive and intrusive phase can, using the new data presented here, be estimated to be of comparable order; and (2) the major and trace element chemistry of the two phases is very similar³⁰. Identical genesis for the two different type of volcanism can, therefore, be concluded, and for simple geometrical reasons, a narrow, rising deep-seated mantle plume cannot account for a dyke swarm like that now known to exist in East Greenland.

I therefore agree with Brown and Whitley³¹, and abandon the hot spot theory. I suggest that the East Greenland Tertiary

volcanism reflects the interactions between the plastic asthenosphere and the brittle fracturing of the lithosphere in connection with incipient continental break-up.

The chemical similarities between the early Tertiary lava province in the Labrador Sea–Baffin Bay area²² and the North Atlantic province has been known for a long time, and the different genesis postulated for the latter has been applied to the former. Palynological (onset of volcanism in Middle Palaeocene (J. M. Hansen, personal communication)) and palaeomagnetic data^{24,25} clearly shows, that at least part of the lava extrusion took place before anomaly 24 simultaneous with the East Greenland volcanism.

Although no dyke swarms occurring on shore in the Labrador–Baffin area resemble those of East Greenland, prominent linear magnetic anomalies in the Davis Strait area, which have been suggested^{28,33–35} to reflect basalts and/or transform faults, may be partly related to major dyke swarms. Oceanic crust is known to exist in the Labrador Sea¹⁶ and Baffin Bay²³. The Davis Strait Sill separates these two oceanic areas and is underlain by Icelandic type crust²³. The plate tectonic evolution of the Labrador–Baffin area has been reviewed by Srivastava²⁸, who concludes that seafloor spreading commenced at anomaly 32 in the Southern Labrador Sea, and at anomaly 28 in the Northern Labrador Sea, whereas he suggested the onset of sea floor spreading in the Davis Strait–Baffin area took place at the time of anomaly 24. The relative motion between Greenland and North America ceased before anomaly 13 (ref. 28).

Interpreting the Davis Strait Sill as thinned continental crust invaded by dykes and overlain by basalt such as those on shore²² (Cape Dyer and Disko), there is a similarity with the North Atlantic at the time of anomaly 24, with seafloor spreading in the south attempting to penetrate northwards, advancing across a continental barrier (Davis Strait) and giving rise to FeTi–basaltic volcanism and re-attaining actual seafloor generation north of the barrier. The magmatic activity in the Labrador–Baffin area and the opening of Baffin Bay is, therefore, suggested to reflect, as in the North Atlantic, the rearrangement of plate movements at the time of anomaly 24,

thereby supporting the most recent interpretation of the Baffin Bay area²⁸.

Finally, it should be noted, that the findings reported here in contrast to the Heirtzler scale⁶, places anomaly 24 at 55–56 Myr which closely agrees with several other authors^{7,8,26,29}.

Financial and logistic support was given by The Geological Survey of Greenland and this paper was written while holding a grant from the Danish Energy Agency. I thank W. S. Watt and C. K. Brooks for improvements to the text.

Received 1 March; accepted 10 May 1978.

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Q β DNA-containing hybrid plasmids giving rise to Q β phage formation in the bacterial host

Tadatsugu Taniguchi, Marta Palmieri & Charles Weissmann

Institut für Molekularbiologie I, Universität Zürich, 8093 Zürich, Switzerland

Hybrid plasmids consisting of PCRI and a complete DNA copy of the RNA genome of coliphage Q β , inserted in either orientation, elicit the formation of phage Q β when introduced into Escherichia coli.

THE availability of a hybrid plasmid containing a complete DNA copy of the RNA of phage Q β from which a biologically competent Q β RNA could be transcribed *in vitro* would greatly facilitate the preparation and study of unconditionally lethal Q β mutants, and would allow genetic manipulations that cannot be carried out at the RNA level. We therefore undertook to synthesise complete Q β DNA by reverse transcription, clone it by hybrid DNA technology and regenerate Q β RNA by transcription of the excised Q β DNA. During these experiments we found that bacteria containing complete Q β DNA hybrid plasmids produced viable Q β phage.

Preparation of a hybrid plasmid containing a complete DNA copy of Q β RNA

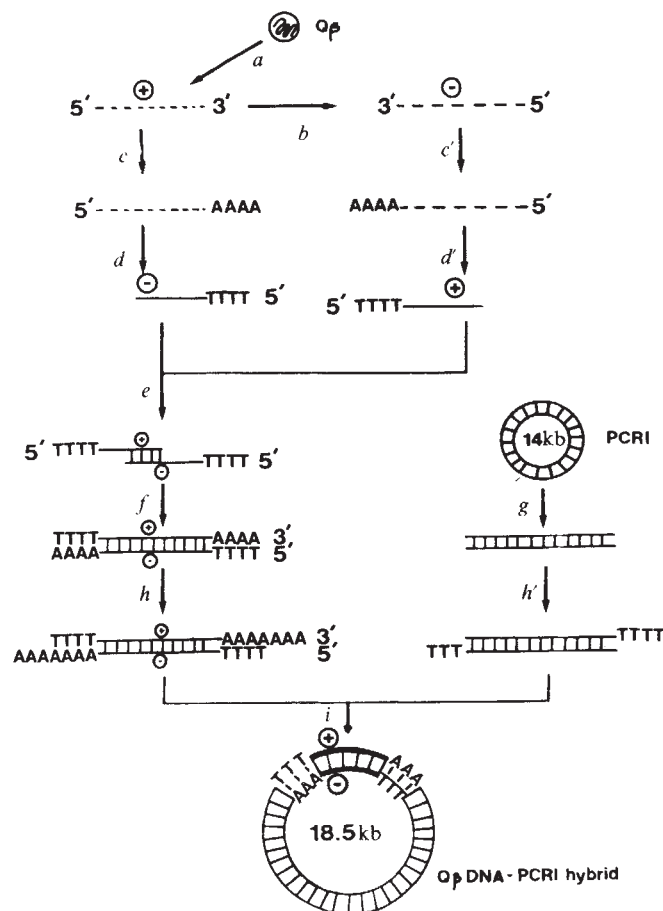
In designing approaches for the preparation of a hybrid plasmid containing a complete copy of the Q β genome, we first considered the potential lethality of the hybrid for the host cell. If the Q β DNA-containing plasmid led to little or no formation of virus and/or deleterious viral products, then wild-type Q β RNA could be used as the template for cDNA synthesis. If the production of virus and/or viral components were lethal for most or all host cells, then the use of RNA from an appropriate temperature-sensitive Q β mutant might allow cloning and propagation of the hybrid plasmid at the nonpermissive, and its expression at the permissive temperature.

We anticipated that the *in vivo* transcription of a viral RNA copy from a Q β DNA plasmid would be inefficient in at least one of the two possible orientations of the insert, and that, moreover, the processing of the primary transcript to yield an

RNA molecule capable of being translated and replicated would be a rare event within the span of the bacterial generation time. We therefore decided to use wild-type Q β RNA as our starting material.

Previous experience had shown that double-stranded cDNA copies of mRNA, prepared by conventional means, lack at least a few nucleotides at the end corresponding to the 5'

Fig. 1 Synthesis of a hybrid plasmid containing a complete DNA copy of the Q β genome. Q β RNA extracted from plaque-purified phage (a) is used as template for the synthesis of Q β minus strands by Q β replicase (b). Q β RNA and purified Q β minus strands are elongated with AMP residues by polyA polymerase (c and c', respectively) and used as templates for reverse transcriptase, with oligo dT as primer (d and d', respectively). The minus and plus cDNA copies are hybridised (e) and the incomplete duplex is repaired with DNA polymerase I (f). Plasmid PCRI is cleaved with *Eco*RI and the ends are trimmed back with 5' exonuclease (g). The double-stranded Q β DNA and the PCRI DNA are elongated with dAMP and dTMP residues, respectively (h and h'), using terminal nucleotidyl transferase. The two components are hybridised and transfected into *E. coli* HB101 (i).



terminus of the RNA (ref. 1). A modified approach, outlined in Fig. 1, was devised to overcome this difficulty. Q β plus and minus strands, synthesised *in vitro* with Q β replicase², were elongated with Ap residues using polyA polymerase³, and separately used as templates for reverse transcriptase in an oligo dT-primed reaction. The cDNA copies were freed of RNA by very mild alkaline digestion followed by sucrose gradient centrifugation. The usual strong alkaline hydrolysis^{4,5} was avoided¹ to minimise the risk of deamination. The chain length of the minus and plus strand Q β DNA was estimated by alkaline agarose gel electrophoresis to range approximately over 3,500–4,500 and 1,600–4,500 nucleotides, respectively (data not shown); Q β RNA has a chain length of approximately 4,500 nucleotides⁶. About equimolar amounts of the complementary strands were hybridised to yield incomplete double strands. Repair synthesis with DNA polymerase I yielded a double-stranded DNA which on agarose gel electrophoresis (Fig. 2) moved as a discrete band with a mobility similar to that of linear pBR322 DNA, which has a chain length of 4,361 base pairs (G. Sutcliffe and W. Gilbert, personal communication).

The double-stranded Q β DNA was elongated with dA residues, hybridised to poly dT-PCRI DNA and transfected into *Escherichia coli* HB101, a strain resistant to infection by Q β phage (data not shown). 612 kanamycin-resistant colonies were generated by 60 ng of the dA-elongated Q β cDNA. 96 kanamycin-resistant clones were gridded and assayed by the Grunstein-Hogness *in situ* hybridisation assay⁷, using Q β [³²P]RNA as probe; 74 gave a positive response. Two sets of the colonies were reassayed by the same procedure, using as probes plus strand and minus strand segments, respectively, ³²P-labelled at the γ position of the 5' terminal pppG residue. As shown in Fig. 3, 32 colonies were labelled by plus, and 22 colonies by minus strand termini; four colonies, ZpQ β -32, ZpQ β -33, ZpQ β -74 and ZpQ β -87, were labelled by both probes.

Properties of *E. coli* HB101 containing Q β DNA plasmids

To test whether any of the clones containing Q β DNA hybrid plasmids were producing Q β phage, colonies of the four clones considered to contain complete Q β DNA copies, as well as clones with Q β DNA which apparently lacked either one or the other terminus, were replica-plated onto a lawn of indicator bacteria (*E. coli* Q₁₃) sensitive to Q β phage. As shown in Fig. 4, three of the four strains containing the complete Q β genome (ZpQ β -32, ZpQ β -33 and ZpQ β -87), but none of the others, gave an area of lysis. Cultures of several bacterial strains were prepared. The bacteria were collected at a cell density of about 10⁸ ml⁻¹ and resuspended in the original volume of medium. The bacterial suspension and the supernatant were titrated on indicator bacteria. As shown in Table 1, the supernatants of the cultures containing the plasmids ZpQ β -32, ZpQ β -33 and ZpQ β -87 had about 5 $\times$ 10⁸ plaque-forming units (PFU) per ml, whereas the value for the bacterial suspensions was about 5 $\times$ 10⁷ PFU per ml. *E. coli* HB101 containing PCRI or plasmid

Table 1 Properties of *E. coli* HB101 containing Q β DNA hybrid plasmids

Plasmid	ZpQ β -32	ZpQ β -33	ZpQ β -74	ZpQ β -87	PCRI
Doubling time (min)	46 $\pm$ 4	40 $\pm$ 0	35 $\pm$ 2	40 $\pm$ 0	36 $\pm$ 3
Bacterial count at A ₆₅₀ 0.8 (cells per ml)	6.7 $\times$ 10 ⁷	8.1 $\times$ 10 ⁷	9.0 $\times$ 10 ⁷	6.0 $\times$ 10 ⁷	1.6 $\times$ 10 ⁸
Plaque-forming units in supernatant (PFU per ml)	5.0 $\times$ 10 ⁸	4.2 $\times$ 10 ⁸	0	3.9 $\times$ 10 ⁸	0
Plaque-forming units in resuspended pellet (PFU per ml)	5.0 $\times$ 10 ⁷	5.7 $\times$ 10 ⁷	0	4.2 $\times$ 10 ⁷	0

Bacteria were grown in tryptone broth containing 25 μ g ml⁻¹ kanamycin at 37 °C, with shaking; the turbidity was measured at 650 nm in a Beckman DB spectrophotometer every 30 min. Growth rates were determined during log phase as average values from two independent experiments. At an apparent absorbance of about 0.8 the bacteria were collected by centrifugation and resuspended in an equal volume of tryptone broth. Bacteria were counted in a Petroff-Hauser counter and plaque-forming units were determined at appropriate dilutions²⁴.

ZpQ β -74 produced no plaque-forming agents. The plaque-forming activity in the supernatant was inhibited by rabbit anti-Q β serum with the same rate constant as phage Q β . The plaque-forming activity of the chloroform-treated but not of the native bacterial suspension was inactivated by the antibody (data not presented), possibly because living cells continued to yield phage after the antibody was diluted out in the plaque assay, whereas chloroform-treated cells did not.

The generation times of the three Q β -producing strains were all greater (40–46 min) than those of *E. coli* HB101 containing PCRI or a nonproducer PCRI-Q β DNA hybrid (36 and 35 min, respectively), showing that Q β production reduced the growth rate of the bacterial population (Table 1). The release of phage could be due to the occasional lysis of a plasmid-containing bacterium, as shown for *E. coli* chronically infected with f₂ phage⁸, or to the constant leakage of a small number of phage from most or all bacteria, as in the case of phage fd-infected *E. coli*^{8,9}. To distinguish between these possibilities *E. coli* HB101 (ZpQ β -32) were washed to remove free phage, and distributed into 96 tubes at a dilution such that each tube contained on average about 0.5 bacteria. As calculated from the Poisson distribution, 60.6% of the tubes should contain no bacteria, and 30, 7.6 and 1.8% of the tubes 1, 2 or 3 and more bacteria, respectively. After incubation, the tubes were scored for bacterial growth and phage content. In a typical experiment, 45 tubes (47%) contained neither phage nor bacteria, 28

tubes contained phage only, and 23 tubes both bacteria and phage; no tubes contained bacteria only (Table 2). It is interesting that the number of phage per tube varied from 10 to 10,000 in the tubes containing phage but no bacteria, and was in general $>10^6$ when bacteria were also present (Table 2).

We interpret these findings as follows. If, on placing a single bacterium in a tube, lysis occurs before the first division, the tube will contain the progeny of a single burst, on average $\sim 1,300$ phage particles¹⁰ and no bacteria. If the bacterium divides, both progeny may lyse to yield a tube with two bursts and no bacteria, one bacterium may lyse and the other divide, or both bacteria may divide. With successive generations the majority of the tubes will be either devoid of bacteria (but contain varying numbers of phage) or they will contain a large population of bacteria (and phage). The mathematical equations describing the events have been derived in connection with Bartholomay's "linear birth and death" model (for a description, see ref. 11).

Physical and biological characterisation of a hybrid plasmid containing Q β DNA

To estimate the sizes and determine the relative orientations of the Q β inserts of the four plasmids ZpQ β -32, ZpQ β -33, ZpQ β -74 and ZpQ β -87, digestions were carried out with *Hind*III, *Eco*RI and both enzymes in combination. As shown

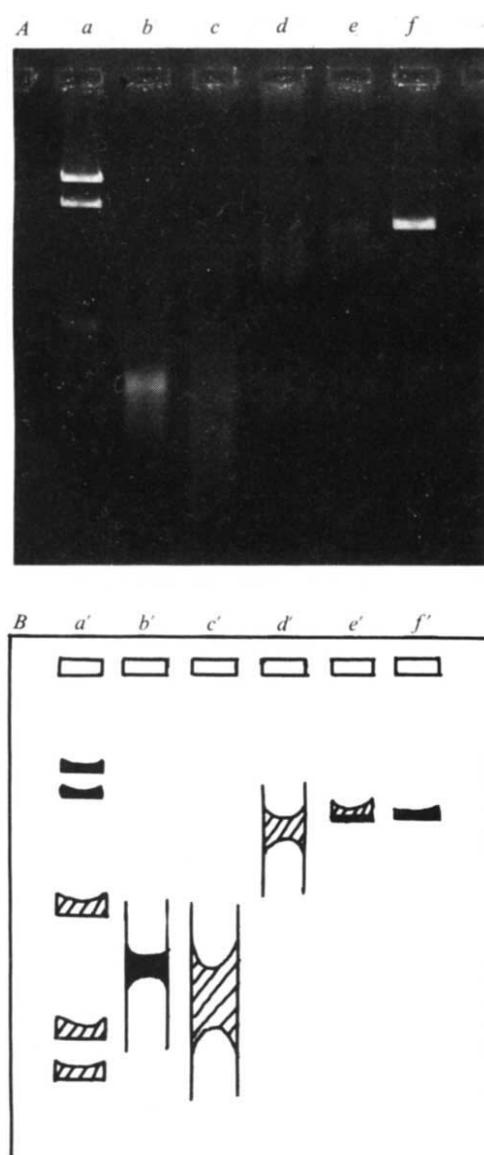


Fig. 2 Agarose gel electrophoresis of Q β DNA synthesised *in vitro*. Q β RNA (200 μ g) extracted²⁵ from plaque-purified Q β phage 'AS' (ref. 14) was used as template for Q β replicase²⁷ (110 units) in a standard synthesis reaction² (final volume 500 μ l) using [α -³²P]ATP (specific activity, 1,300 c.p.m. nmol⁻¹) as labelled substrate. The product (216 nmol AMP incorporated, about 300 μ g RNA) was deproteinised and the minus strands were purified² (final yield, 47 μ g). 190 μ g of Q β plus and 43 μ g of minus strands were incubated with 127 and 25 units, respectively, of polyA polymerase from *E. coli* in standard assay conditions²⁸ using ³H-labelled ATP (0.23 mM, specific activity, 3.4×10^4 c.p.m. nmol⁻¹). The products were purified by phenol extraction, Sephadex G-100 chromatography and ethanol precipitation. To prepare Q β cDNA minus and plus strands, polyA-Q β plus strands (50 μ g) and polyA-Q β minus strands (36 μ g), respectively, were incubated for 90 min at 37°C in 40 mM Tris-HCl (pH 7.5), 30 mM NaCl, 5 mM MgCl₂, about 0.5 mM each of dGTP, dCTP, dTTP and 0.44 mM [³H]dATP (specific activity, 1.1×10^5 c.p.m. nmol⁻¹) with 6 μ g and 4 μ g, respectively, of oligo dT, and 75 and 55 units, respectively, of purified AMV reverse transcriptase²⁹. The products were deproteinised with phenol, passed through Sephadex G-100, precipitated with ethanol and digested for 40 min at room temperature in 50 μ l 0.5 M KOH. The samples were diluted with 200 μ l water and centrifuged in a SW60 rotor for 2.5 h at 55,000 r.p.m. and 4°C through a 5–23% neutral sucrose gradient in 50 mM Tris-HCl (pH 7.5). The fractions containing the fast sedimenting DNA were precipitated with ethanol: 8.7 μ g of cDNA minus and 3.5 μ g of cDNA plus strands were recovered. 1 μ g each of plus and minus strand cDNA were annealed for 21 h at 20°C in 320 μ l 50% formamide, 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.5 mM EDTA. After extensive dialysis against 1 mM Tris-HCl (pH 8.0), the solution was concentrated *in vacuo* to 15 μ l (yield, 1.8 μ g DNA). The preparation was adjusted to 110 mM potassium phosphate (pH 6.9), 10 mM MgCl₂, 10 mM dithiothreitol, 1 mM each of dATP, dGTP, dTTP, 0.95 mM [α -³²P]dCTP (specific activity, 3.26×10^5 c.p.m. nmol⁻¹) and incubated with 27 units of DNA polymerase I of *E. coli* (Boehringer) (final volume, 40 μ l) for 7 h at 15°C. After extraction with phenol, the DNA was purified by chromatography on Sephadex G-100. DNA samples in 20 μ l of Loening buffer³⁰ were applied to the slots of a 8 $\times$ 9.5 $\times$ 0.5 cm horizontal 1% agarose gel and run in Loening buffer for 3 h at 50 mA. The gel was stained with 1 μ g per ml ethidium bromide. A, The gel was photographed under ultraviolet illumination. a, ϕ 29 DNA³¹ cleaved with endonuclease *Eco*RI (ref. 32); b, 100 ng cDNA minus strands; c, 200 ng DNA plus strands; d, 200 ng annealed minus and plus strand cDNA; e, as d after repair synthesis with DNA polymerase I (40 ng); f, pBR322 DNA³³ (20 ng) cleaved with endonuclease *Eco*RI. B, Tracing of A.

Table 2 Analysis of phage production and population-forming capacity of single *E. coli* HB101 containing the Q β -inducing hybrid plasmid ZpQ β -32

Bacteria (colony-forming units per tube)		Phage (PFU per tube)							
		0	10 ⁰ -10 ¹	10 ¹ -10 ²	10 ² -10 ³	10 ³ -10 ⁴	10 ⁴ -10 ⁵	10 ⁵ -10 ⁶	>10 ⁶
	0	45		4	14	9	1		
	10 ⁰ -10 ³							1	1
	10 ³ -5 $\times$ 10 ³								12
	>5 $\times$ 10 ³						1		8

E. coli HB101 containing plasmid ZpQ β -32 were grown in tryptone broth to a cell density of 7×10^7 cells per ml, as determined by counting in a Petroff-Hauser chamber, and diluted in tryptone broth to 5 cells per ml. 100- μ l aliquots were distributed into 96 wells of a microtitre plate (Nunc) and incubated for about 20 h at 37 °C. Plaque-forming units were determined by plating appropriate dilutions on a lawn of *E. coli* Q₁₃ and bacterial titres were estimated by determining colony-forming units on kanamycin-containing agar plates. There is an inherent error in this determination due to the reduced colony-forming capacity (45%) of this strain. In a control experiment, *E. coli* HB101 was distributed into 192 wells (on average, 0.5 cells per well) in a similar fashion: 71 wells (37%) showed bacterial growth (expected value, 39%).

in Fig. 5, *Hind*III and *Eco*RI each cleaved the hybrid DNA ZpQ β -32 at one site. PCR1 contains one site each for *Hind*III and *Eco*RI (ref. 12); as the Q β DNA was inserted into the *Eco*RI site by the AT-tail method this site was obliterated and the *Eco*RI site of the hybrid must be within the Q β sequence. Cleavage of the hybrid plasmids with *Eco*RI and *Hind*III yielded two fragments: about 4,700 and 13,000 base pairs in the case of ZpQ β -32 and ZpQ β -33 and about 7,700 and 10,000 base pairs in the case of ZpQ β -74 and ZpQ β -87. As the *Eco*RI site is known to lie approximately 1,000 nucleotides from the 5' end of Q β RNA (P. Mekler, unpublished results), the orientation of the inserts is as indicated in Fig. 6.

The specific infectivity of the Q β DNA plasmid 32 (that is, its capacity for eliciting Q β production on transfection) was compared to that of Q β RNA in an infectious centre assay, using *E. coli* K12W6 spheroplasts. As shown in Table 3, the specific infectivity of the plasmid was almost equal to that of the RNA on a weight basis, and seven times greater on a molar basis. Treatment with restriction enzyme *Bsp*I (an isoschizomer of *Hae*III (ref. 13)) abolished the Q β -producing activity of the plasmid, but not that of Q β RNA, whereas the opposite result was found with RNase A. No plaques were obtained when 1 μ g of plasmid DNA was plated on *E. coli* Q₁₃. These experiments show that the plasmid DNA, and not contaminating Q β RNA

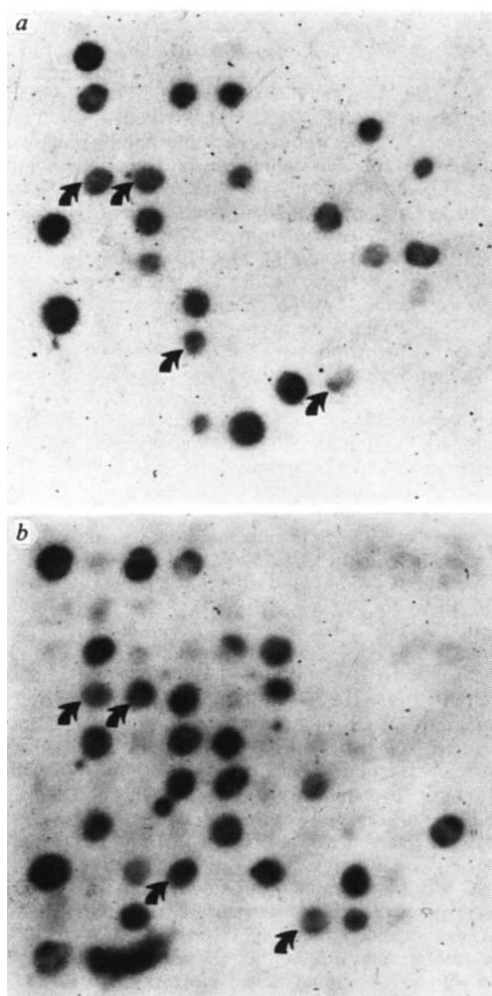


Fig. 3 Identification of hybrid plasmid clones containing the DNA sequence corresponding to, *a*, the 3' and, *b*, the 5' terminus of Q β RNA. 0.2 μ g of the double-stranded Q β cDNA described in the legend to Fig. 2 were incubated with 6 units terminal nucleotidyl transferase³⁴ in 100 mM sodium cacodylate (pH 7.2), 10 mM NaH₂PO₄, 5 mM MgCl₂, 1 mM dATP, 50 μ g per ml BSA for 60 min at 37 °C (final volume, 20 μ l). Poly dT-PCR1 was prepared by cleaving PCR1 DNA¹² with endonuclease *Eco*RI (ref. 32), digesting it with 5' exonuclease³⁵ to remove about 20 nucleotides per end and elongating it with dTMP residues using terminal nucleotidyl transferase³⁴. 20 ng of poly dA-Q β DNA and 100 ng of poly dT-PCR1 were annealed in 50 μ l 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA for 2 h at 47 °C, 1 h at 37 °C and 1 h at 20 °C. Transfection³⁶ was into *E. coli* HB101. This strain cannot be infected naturally with Q β phage, but it supports Q β phage replication when Ca²⁺-treated cells are transfected with Q β RNA (unpublished observations). 20 kanamycin-resistant colonies per ng of DNA were obtained, of which about 80% contained Q β -specific sequences as determined by the colony hybridisation assay of Grunstein-Hogness⁷, using Q β [³²P] RNA (4×10^6 c.p.m. μ g⁻¹) as probe (data not shown). 96 clones were gridded, replica-plated onto two Millipore filters and subjected to the colony-hybridisation assay using γ -³²P-labelled Q β minus strand termini (*a*) or γ -³²P-labelled Q β plus strand termini (*b*). γ -³²P-labelled minus strand termini were prepared by incubating 9.6 μ g of Q β RNA with 8.8 units Q β replicase and 12 units host factor containing 100 μ M ATP, 80 μ M [γ -³²P]GTP (ref. 37) (specific activity, 3×10^8 c.p.m. nmol⁻¹) in standard conditions². After 10 min at 37 °C UTP and CTP were added at 70 μ M, and incubation was continued for 20 s. The product (1.4×10^5 c.p.m.) was isolated by phenol extraction, Sephadex G-25 chromatography and ethanol precipitation. Plus strand termini were synthesised similarly, however, using 4.4 μ g minus strand as template and omitting host factor (yield, 1.3×10^5 c.p.m.). The RNA probes were denatured and hybridised to the colony-bearing filter (8 cm diameter) in 1 ml of 50% formamide, 20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 0.5% SDS and 100 μ g per ml yeast RNA, for 8 h at 37 °C and 12 h at room temperature under paraffin oil. The filters were washed with CHCl₃, 50% formamide, 6 $\times$ SSC, 2 $\times$ SSC and digested for 30 min with 200 units per ml of RNase T₁ in 2 $\times$ SSC, all at 20 °C. After exhaustive rinsing with 2 $\times$ SSC, the filter was dried and autoradiographed at -70 °C, using an Ilford intensifier screen. Four colonies (indicated by arrows) responded to both probes.

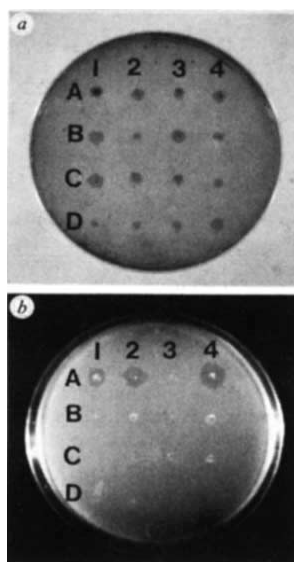


Fig. 4 Identification of phage-producing colonies of *E. coli* HB101 transfected with Q β DNA hybrid plasmids. The four bacterial clones believed to contain complete Q β DNA (see Fig. 3) as well as 12 clones with incomplete copies were inoculated onto agar plates. The colonies were replica-plated onto an agar plate freshly covered with soft agar containing about 5×10^7 *E. coli* Q β , a strain susceptible to infection by phage Q β . Positions A-1 to A-4 are occupied by colonies containing ZpQ β -32, ZpQ β -33, ZpQ β -74 and ZpQ β -87, respectively, positions B-1 to D-4 by colonies containing incomplete Q β DNA plasmids. *a*, Colonies before replica-plating; *b*, colonies replica-plated onto a lawn of indicator bacteria and incubated overnight.

or Q β phage, is responsible for eliciting Q β phage formation. In a further experiment, *E. coli* HB101 was infected with the hybrid plasmid ZpQ β -32 and kanamycin-resistant clones were screened for Q β phage production. All of 100 clones examined produced phage as determined by replica-plating the colonies onto a lawn of indicator bacteria (data not shown). Thus, within about 40 bacterial generations (the number required to expand a single bacterium to the culture from which the DNA was prepared), less than 6×10^{-8} mutations lethal to Q β occurred per nucleotide and doubling. It remains to be seen whether on prolonged culturing there is a selection for bacteria containing a reduced number of Q β hybrid plasmids capable of yielding infectious Q β RNA.

Characterisation of the Q β RNA formed following infection with Q β DNA-containing plasmids

Multipli-passaged Q β phage populations contain a high proportion of viable variants¹⁴. Although the Q β RNA used to generate the Q β DNA plasmids was prepared from plaque-purified virus, it was of interest to compare the T₁ fingerprints of authentic wild-type Q β RNA and the RNA of phage recovered from Q β DNA plasmid-infected cells. ³²P-labelled RNA of phage derived from cells infected with the hybrid plasmids ZpQ β -32, ZpQ β -33 and ZpQ β -87 as well as wild-type Q β [³²P]RNA was treated with RNase T₁ and the oligonucleotides were separated by two-dimensional polyacrylamide gel electrophoresis¹⁵. All autoradiograms were indistinguishable, and identical with that shown in Plate 3(a) of ref. 14, confirming the identification of the virus as 'wild type' Q β . In particular, the presence of the 3' terminal T₁-resistant oligonucleotide T-18 showed that the 3' end of the plasmid-induced phage RNA was identical with that of the wild-type RNA.

Discussion

The synthesis of a DNA copy of the Q β genome involved the use of Q β replicase, AMV reverse transcriptase and *E. coli* DNA polymerase I, the first two of which are reported to have a high error rate, of about 10^{-4} errors, respectively, per nucleotide and doubling^{16,17}. Nonetheless, three out of four hybrid plasmids which contained a complete copy of the Q β genome (as evidenced by hybridisation of the termini) proved capable of eliciting Q β phage formation in *E. coli*. This proportion may be a low estimate, as the colony-forming capacity of Q β -producing transformants is only about 45% (see Table 2).

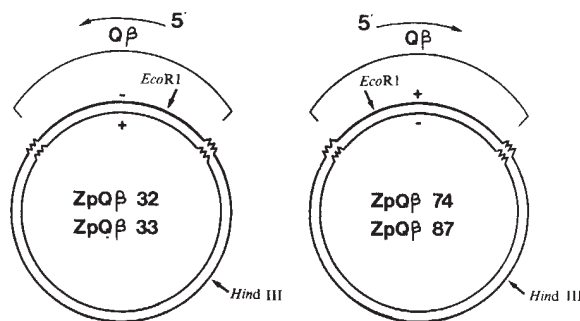
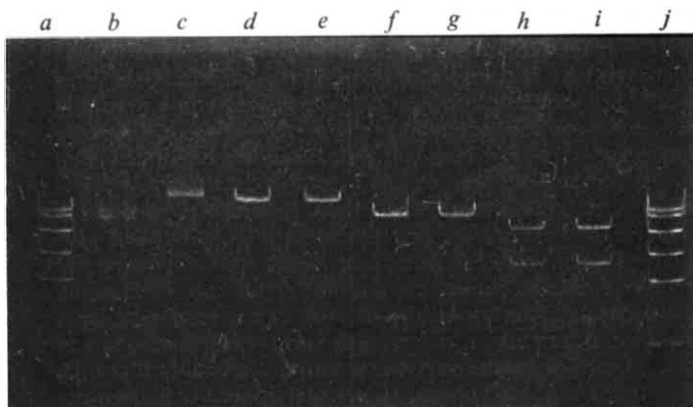
E. coli propagating a Q β DNA genome and producing Q β phage constitute a novel biological system, although superficially they resemble a population chronically infected with Q β phage, where the bulk of the bacteria replicate normally, a certain fraction lyses, releasing phage particles, and the population is largely resistant to infection from outside, that is, does not permit plaque formation when used as lawn^{8,9,18-20}. The exact mechanism of this so-called 'carrier-state' is not known; however, it is probably based on horizontal⁸ and/or vertical²⁰ transmission of the phage RNA genome in conditions which (1) preclude efficient viral expression and (2) reduce susceptibility to infection. There is no evidence that DNA of *E. coli* acutely infected with an RNA phage contains

Table 3 Specific infectivity (Q β formation) of ZpQ β -32 DNA or Q β RNA by the infectious centre assay

Nucleic acid added, ng		Buffer	Treatment		Plaques found	PFU μg^{-1}
			<i>Bsp</i> I	RNase A		
Q β RNA	10	0.5 mM EDTA	—	—	250	2.5×10^4
	400	Y-50	—	—	1062	2.7×10^3
	400	Y-50	+	—	1186	3.0×10^3
	400	Y-50	—	+	6	15
ZpQ β -32 DNA	10	20 mM Tris-HCl	—	—	208	2.1×10^4
	10	Y-50	—	—	165	1.7×10^4
	10	Y-50	+	—	0	0
	10	Y-50	—	+	233	2.3×10^4

Spheroplasts of *E. coli* K12W6 were prepared and infected as described¹⁰, and plated on lawns of *E. coli* Q β . Q β RNA was prepared by the method of Weissmann *et al.*²⁵. ZpQ β -32 DNA was purified by a modification of the procedure of Currier and Nester²⁶; the yield was 500 μg form I DNA per l of culture. 0.5 μg DNA or 20 μg RNA were treated with 5 units of *Bsp*I restriction enzyme¹³ in 25 μl Y-50 buffer (10 mM Tris-HCl (pH 7.5), 6 mM MgCl₂, 50 mM NaCl, 6 mM β -mercaptoethanol, 200 μg per ml bovine serum albumin (BSA)), or with 1 ng pancreatic RNase A in the same buffer for 30 min at 37 °C. After the reaction, 25 μl of a solution containing 0.93 mg ml⁻¹ self-digested Pronase and 0.2% sodium dodecyl sulphate were added and incubation was carried out for 30 min at 37 °C. 5 μl were withdrawn and mixed with 20 μl of 20 mM Tris-HCl (pH 7.5). Of this, 5 μl (corresponding to $\frac{5}{50}$ of original material) were diluted with 0.2 ml 0.5 mM EDTA, mixed with spheroplasts and plated on *E. coli* Q β as described¹⁰.

Fig. 5 Restriction analysis of Q β DNA-containing hybrid plasmids. Cultures of *E. coli* HB101 containing plasmids ZpQ β -32, ZpQ β -33, ZpQ β -74 and ZpQ β -87 were grown to about 10^8 cells per ml, chloramphenicol (0.17 mg ml^{-1}) was added and plasmid DNA was purified²⁶ after 12 h further incubation. Digestions with *EcoRI* and *HindIII* were carried out in 10 mM Tris-HCl (pH 7.5), 6 mM MgCl₂, 6 mM β -mercaptoethanol, 200 μg per ml bovine serum albumin containing 100 mM NaCl (*EcoRI*) or 50 mM NaCl (*HindIII*). Electrophoresis was on a vertical $165 \times 165 \times 3 \text{ mm}$ 1% agarose slab gel in Loening buffer³⁰, at 120 mA for 5.5 h. After staining with $1 \mu\text{g ml}^{-1}$ ethidium bromide, the gel was photographed under ultraviolet illumination. *a*, Size marker mixtures of PCRI singly cleaved with *EcoRI* (13,000 base pairs), *PstI* (11,850 base pairs, 1,150 base pairs) and *KpnI* (7,470 base pairs, 5,570 base pairs) and doubly-cleaved with *EcoRI* and *HindIII* (9,200 base pairs, 3,800 base pairs). The sizes of the marker fragments were determined by calibration of their cleavage products against appropriate pBR322 fragments of known length (G. Sutcliffe and W. Gilbert, personal communication). *b*, PCRI form I. *c*, ZpQ β -32 form I. *d*, ZpQ β -32 cleaved by *HindIII*. *e*, ZpQ β -32 cleaved by *EcoRI*. *f*, ZpQ β -32; *g*, ZpQ β -33; *h*, ZpQ β -74; *i*, ZpQ β -87 cleaved by *EcoRI* and *HindIII*. *j*, as *a*.



We thank Dr E. Domingo for help in the early phases of this work, Dr A. Kiss for restriction enzyme *BspI* and Dr S. Clarkson for *HindIII*. The research was supported by grants of the Kanton of Zürich and the Schweizerische Nationalfonds (no. 3.475.75).

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Neoplastic transformation induced by a direct perturbation of DNA

J. Carl Barrett*, Takeki Tsutsui & Paul O. P. Ts'o

School of Hygiene and Public Health, Johns Hopkins University, Baltimore, Maryland 21205.

Treatment of Syrian hamster embryo cells with 5-bromodeoxyuridine followed by near ultraviolet irradiation results in neoplastic transformation of these cells. This demonstrates that a direct perturbation of DNA is sufficient to initiate neoplastic transformation.

THE critical cellular target in chemical carcinogenesis has not been identified unequivocally, although existing evidence implicates DNA damage as an important step in neoplastic transformation. Experiments by Brookes and Lawley¹ indicated a correlation between carcinogenic potential and binding of polycyclic aromatic hydrocarbons to DNA of mouse skin. Further evidence for an association between carcinogenicity and perturbation of DNA is provided by the higher rate of development of malignant neoplasms in patients with hereditary diseases. Xeroderma pigmentosum²⁻⁵ individuals, who lack the ability to repair DNA damage induced by ultraviolet irradiation and certain chemicals, and ataxia telangiectasia^{6,7} patients, who are unable to repair DNA damage caused by ionising radiation, have a marked tendency to develop malignant disease. Additionally, a good correlation exists between the carcinogenicity and mutagenesis of many chemical carcinogens⁸. Although several prevalent theories of carcinogenesis, as well as various short-term tests for carcinogens, are predicated on the assumption that DNA is the critical target in chemical induction of neoplasia, there is no direct proof for this assumption.

A direct demonstration of the role of DNA damage in chemically induced neoplastic transformation is difficult, as most chemical carcinogens, when activated metabolically, are reactive electrophiles which bind covalently to many cellular macromolecules, including DNA, RNA and proteins⁹. Therefore, rather than study the interaction of known carcinogens with cellular macromolecules, we have investigated whether a direct perturbation of DNA is sufficient to initiate neoplastic transformation. The direct perturbation was accomplished by treatment of cells in culture with 5-bromodeoxyuridine (BrdU) and near ultraviolet light (near UV). BrdU is incorporated exclusively into cellular DNA in place of its analogue, thymidine. Such BrdU-substituted DNA exhibits an ultraviolet absorption spectrum which is shifted toward longer wavelengths; consequently, irradiation with light of wavelengths >300 nm (near UV) produces a significantly higher number of photochemical lesions in BrdU-substituted DNA than occurs in nonsubstituted DNA. Current findings suggest that the major photochemical reaction of BrdU-substituted DNA is an initial photodissociation of the bromine atom, producing an uracil radical, followed by subsequent decomposition of this radical¹⁰. In alkaline conditions, DNA single-strand breaks are observed. Biological consequences of treatment with BrdU followed by near UV irradiation include chromosome damage¹¹, cytotoxicity¹⁰ and somatic mutations¹².

Syrian hamster embryo (SHE) cells are widely used for quantitative studies of neoplastic transformation induced by chemical carcinogens^{13,14}. We report here that neoplastic transformation of these cells occurs on treatment with BrdU followed by near UV irradiation.

Cytotoxicity and single-strand breaks induced by BrdU treatment plus near UV irradiation

Treatment with BrdU alone was cytotoxic to early passage SHE cells grown as described in Fig. 1. The population doubling time of SHE cells was approximately 15 h; therefore, a 24-h treatment with BrdU encompassed at least one cell cycle. Treatment of the cells with 10^{-6} M BrdU for 24 h resulted in 70% lethality, even when the experiments were carried out under illumination by red light only. Meuth and Green¹⁵ have reported that the cytotoxicity of BrdU is attributable to a deoxycytidine-less state caused by the allosteric inhibition of ribonucleotide reductase. We examined the effect of exogenous deoxycytidine (dC) on the cytotoxicity of BrdU and found that it partially, though not completely, reduced the lethality (Fig. 1). For this reason, 2×10^{-4} M dC was included in all other experiments.

Irradiation of SHE cells with near UV light after BrdU treatment significantly enhanced the induced lethality. Irradiation alone for five minutes was not cytotoxic and treatment with BrdU (10^{-6} M, 24 h) in the absence of irradiation resulted in a surviving fraction of 0.4, whereas five minutes of irradiation after BrdU treatment resulted in a surviving fraction of 0.03. Cytotoxicity increased with increasing dose of BrdU or near UV irradiation.

Treatment with BrdU and irradiation also resulted in single-strand breaks in cellular DNA, detectable by alkaline sucrose gradient analysis (Fig. 2). BrdU (10^{-6} M, 3 h) alone did not cause measurable DNA strand breakage, whereas BrdU incorporation followed by 1–30 min of near UV irradiation did result in a reduction of the sedimentation coefficient of the DNA, indicative of single-strand breakage (Fig. 2). The extent of breakage increased with increasing dosage of irradiation.

Somatic mutations induced by BrdU treatment plus near UV irradiation

BrdU treatment and subsequent near UV irradiation was also mutagenic to SHE cells, causing measurable somatic mutations at two genetic loci. Mutations affecting either hypoxanthine phosphoribosyl transferase (EC2.4.2.8) (HPRT) or (Na^+ , K^+) ATPase (EC3.6.1.3) function were quantitated by selection of 6-thioguanine or ouabain resistant colonies, respectively¹⁶. A maximum induced frequency of mutation was observed following an expression time of seven days (Table 1). Treatment with BrdU alone (3×10^{-6} M, 24 h) or near UV irradiation alone (5 min) was not mutagenic. In contrast, combined BrdU (3×10^{-6} M, 24 h) and irradiation treatment was mutagenic and the induced mutation frequency increased with increasing dose of irradiation (1–5 min). Isolated mutant colonies retained their resistant phenotype following over 15 population doublings in the absence of the selective agent. 6-Thioguanine-resistant colonies demonstrated cross-resistance to 8-azaguanine, possessed less than 1% of the wild-type HPRT activity, and demonstrated a low reversion frequency ($<10^{-6}$) as measured by growth in hypoxanthine, aminopterin and thymidine medium¹⁶. Ouabain-resistant colonies exhibited a decreased sensitivity to ouabain inhibition of rubidium (^{86}Rb) uptake¹⁶.

Although treatment with BrdU alone has been reported to be mutagenic to mammalian cells¹⁷, the concentration required

* Present Address: National Institute of Environmental Health Sciences, National Institutes of Health, PO Box 12233, Research Triangle Park, North Carolina 27709.

to induce mutation exceeded that used in these experiments. Our results indicate that treatment with 3×10^{-6} M BrdU and subsequent near UV irradiation can induce somatic mutation at the HPRT and $(\text{Na}^+, \text{K}^+)$ ATPase loci, whereas neither treatment alone was sufficient to induce detectable mutations.

Morphological transformation induced by BrdU treatment plus near UV irradiation

As BrdU treatment plus near UV irradiation of SHE cells is cytotoxic, produces single-strand breaks in the DNA and induces somatic mutations, we also examined whether this treatment could induce neoplastic transformation; this can be quantitated by the number of morphologically transformed colonies induced by a given treatment^{13,14}.

Control experiments (no treatment, treatment with 2.5×10^{-6} M BrdU alone, and treatment with up to 20 min near UV irradiation alone) did not induce significant morphological

Fig. 1 Cytotoxicity of BrdU treatment plus near UV irradiation. Cell cultures were established from 13-d gestation fetuses collected aseptically by caesarian section from inbred Syrian hamsters, strain LSH/ss LAK (Lakeview Hamster Colony, Newfield, New Jersey) and pools of primary cultures from littermates were stored in liquid nitrogen. Secondary cultures were initiated from the frozen stocks and all experiments were carried out with tertiary cultures grown in humidified 5% CO_2 at 37°C . The cell culture medium used was IBR modified Dulbecco's Eagle's reinforced medium (Biolabs) supplemented with 0.22% NaHCO_3 and 10% Rehatutin fetal bovine serum (Reheis) without antimicrobial agents. Cells were transferred by a gentle trypsinisation with 0.1% trypsin solution (1:250, Gibco) for 5 min at 37°C . All cells were tested periodically by Microbiological Associates, and found free of mycoplasma contamination. To assay cytotoxicity, $5\text{--}10 \times 10^3$ cells were plated overnight in 100-mm plastic Petri dishes in normal growth medium. In the specified culture, 2×10^{-4} M deoxycytidine (dC) was added to the growth medium for the duration of the experiment. After attachment, medium containing the specified concentration of BrdU was added and the cells were incubated for 24 h. After this time, cell cultures were washed twice with 5 ml of phosphate buffered saline (PBS). The cells were then irradiated in 5 ml of PBS at room temperature with six BLB fluorescent bulbs (Westinghouse) at a distance of 7.5 cm ($\sim 330 \text{ erg}^{-1} \text{ mm}^{-2} \text{ s}^{-1}$ as determined by potassium ferrioxalate actinometry) for the specified time. Control plates were treated identically and held for the same time at room temperature. All procedures were carried out in a room illuminated by only red light. After treatment, the normal growth medium was added to the plates and the cells were allowed to form colonies for 7–8 d. The colonies were fixed with methanol and stained with 10% aqueous Giemsa solution. The number of colonies was then counted and the relative cell survival expressed as the number of colonies in the treated control plates divided by the number in the untreated control plates. The cloning efficiency of the control cells was 4–6%. Cells were treated with BrdU alone ($\circ$), BrdU+dC alone ($\bullet$), BrdU+dC+1 min near UV light ($\square$), BrdU+dC+3 min near UV light (∇), or BrdU+dC+5 min near UV light ($\triangle$).

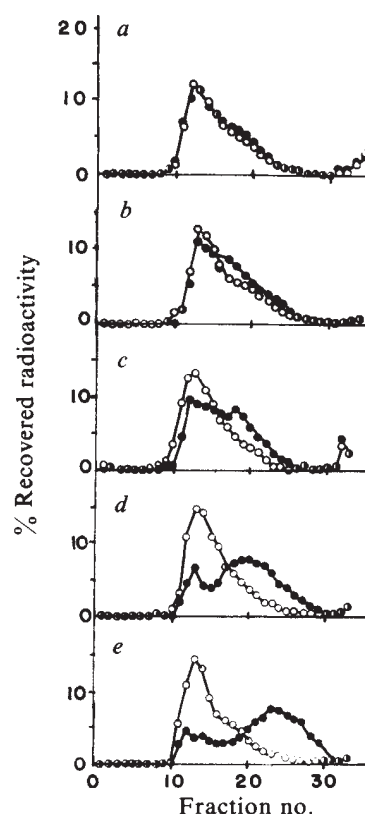
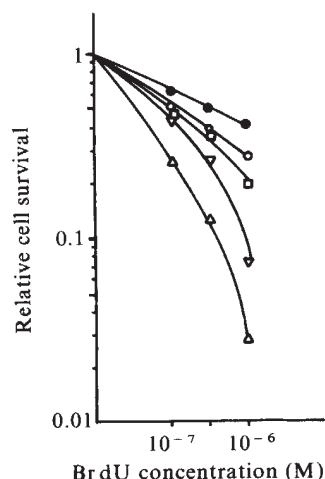


Fig. 2 DNA single strand breaks induced by BrdU plus near UV irradiation. Syrian hamster embryo cells were grown overnight in the presence of $0.1 \mu\text{Ci}$ per ml ^{14}C -thymidine (55 mCi mmol^{-1}). After a 1 h chase, the cells were incubated for 3 h in a medium containing $3 \mu\text{Ci}$ per ml ^3H -thymidine (55 Ci mmol^{-1}), 5×10^{-7} M thymidine and 10^{-6} M BrdU. Following the treatment, the cells were washed twice with PBS, chased in a medium containing 10^{-6} M thymidine for 1 h, and irradiated for 0(a), 1(b), 3(c), 10(d) and 30 min (e). After trypsinisation at 0°C , $\sim 10^4$ cells were lysed for 1 h at 37°C on top of 5–20% alkaline sucrose gradients and then centrifuged in a Beckman SW 50.1 rotor at 34,000 r.p.m. for 90 min at 12°C , as previously described^{20,21}. The molecular weight of DNA labelled with ^{14}C -thymidine was $\sim 2 \times 10^8$ (refs 21, 22). Sedimentation from right to left. $\circ$, DNA profile labelled with ^{14}C -thymidine; $\bullet$, DNA profile labelled with ^3H -thymidine.

transformation of SHE cells (2 out of 7,500 clones), but morphological transformation did occur on treatment with BrdU for 24 h followed by near UV irradiation. Following treatment with 2.5×10^{-6} M BrdU, one-minute irradiation resulted in 1.2% morphological transformation of the surviving colonies. Up to 5% of the surviving colonies were morphologically transformed following 2.5×10^{-6} M BrdU treatment plus five minutes irradiation (Table 2). As shown in Fig. 3, the frequency of transformation increased with increasing doses of BrdU or irradiation. Treatment of the cells with 3×10^{-5} M thymidine during 2.5×10^{-6} M BrdU treatment almost completely abolished the cytotoxicity and morphological transformation effects (data not shown).

Neoplastic transformation induced by BrdU treatment plus near UV irradiation

BrdU plus irradiation also induced neoplastic transformation of SHE cells. Mass cultures of cells were treated with near UV irradiation alone, BrdU alone or BrdU plus near UV irradiation. In two experiments, duplicate cultures were subjected to each of these treatment conditions. All cultures were passaged continually and tested for growth in soft agar^{18,19} and tumorigenicity at various times following treatment.

All untreated cultures senesced by passage 20 and did not produce cells capable of growth in agar. These cells were

nontumorigenic between passages 5–20 on subcutaneous injection of 10^7 cells into newborn hamsters. Identical results were obtained for cultures treated with BrdU (2.5×10^{-6} M) alone (Table 3). Of four cultures treated with near UV irradiation only, one transformed and three senesced. Cells from the transformed culture escaped senescence, formed

colonies in soft agar and were tumorigenic. This transformation occurred at the 40th passage after treatment. This culture had received five minutes near UV irradiation; a parallel culture treated identically did not produce transformants. Moreover, in a second experiment, two cultures treated with near UV irradiation for 20 min did not transform (Table 3).

Table 1 Somatic mutations of SHE cells induced by treatment with BrdU plus near UV irradiation

BrdU concentration (M)	Time of irradiation (min)	Expression time (d)	Relative cloning efficiency (%)	Corrected mutation frequency	
				6TG ^r	Oua ^r
0	0	0	100	$<10^{-6}$	$<10^{-6}$
0	0	4	100	$<10^{-6}$	$<10^{-6}$
0	0	7	100	$<10^{-6}$	$<10^{-6}$
0	0	10	100	$<10^{-6}$	$<10^{-6}$
3×10^{-6}	3	4	55	9.2×10^{-6}	2.2×10^{-6}
3×10^{-6}	3	7	84	4.2×10^{-5}	5×10^{-5}
3×10^{-6}	3	10	90	3.5×10^{-5}	2.7×10^{-5}
0	0	7	100	$<10^{-6}$	$<10^{-6}$
0	5	7	100	$<10^{-6}$	$<10^{-6}$
3×10^{-6}	1	7	100	6.6×10^{-6}	5.2×10^{-5}
3×10^{-6}	3	7	80	3.2×10^{-5}	4.8×10^{-5}
3×10^{-6}	5	7	75	3.5×10^{-5}	6.9×10^{-5}

Somatic mutation of SHE cells was measured as previously described¹⁶. SHE cells were allowed to attach overnight and then treated with BrdU and deoxycytidine (2×10^{-4} M) for 24 h. The cultures were then irradiated as indicated and the cells allowed to grow for four days in normal growth medium. They were then subcultured and a portion of the cultures was assayed by selection for mutants capable of growth in $5 \mu\text{g}$ per ml 6-thioguanine (6TG^r) or 1.25 mM ouabain (Oua^r). The other portion of the culture was grown in normal growth medium for an additional three days (seven days' expression time), subcultured and then assayed for 6TG^r and Oua^r colonies. This process was continued to permit 10 days' total expression time before selection. The selection was measured in duplicate or triplicate by growing 10^6 total cells in medium containing 6TG or ouabain at a density of 10^5 cells per 100-mm dish for 14 days. The number of resistant colonies was determined after fixing and staining. A parallel assay for relative survival was carried out by cloning the cells at low density in normal growth medium. The mutation frequency was calculated as described by correcting for cytotoxicity and the recovery efficiency of the mutants, which was 18–20% (ref. 16).

Table 2 Transformation of Syrian hamster embryonic fibroblasts induced by BrdU treatment followed by near UV irradiation

Treatment	Irradiation time (min)	Survival	% Morphological transformation
None		100	0 (0/2,803)
Irradiation only	3	100	0.04 (1/2,764)
	20	80	0 (0/527)
BrdU only (2.5×10^{-6} M)		50	0.07 (1/1,442)
BrdU + irradiation (2.5×10^{-6} M)	1	14.9 (29.7)	1.2 (8/654)
	3	7.6 (15.1)	3.4 (10/290)
	5	1.5 (3)	5.0 (8/159)

Cells were grown and treated as described in the legend to Fig. 1. The % survival was calculated relative to the survival of the untreated controls. The numbers in parentheses are the % survival of the BrdU + irradiation treated cultures relative to the cultures treated only with BrdU. The % morphological transformation was determined from the number of colonies with an altered morphology^{13,14} relative to the total surviving colonies $\times 100$. The numbers in parentheses are the number of transformed colonies per total number of colonies examined.

Table 3 Neoplastic transformation of SHE cells induced by BrdU plus near UV light treatment

Treatment	Growth in soft agar		Tumorigenicity	
	Culture 1	Culture 2	Culture 1	Culture 2
Experiment 1				
None	—	—	—	—
Near UV only, 5 min	+	—	+	—
BrdU only	—	—	—	—
BrdU plus 1 min near UV	+	—	+	—
BrdU plus 3 min near UV	—	+	—	+
Experiment 2				
None	—	—	—	—
Near UV only, 20 min	—	—	—	—
BrdU only	—	—	—	—
BrdU plus 1 min near UV	—	—	—	—
BrdU plus 3 min near UV	+	+	+	+

Mass cultures of SHE cells were treated with 2.5×10^{-6} M BrdU for 24 h and irradiated with near UV light as specified. In each experiment, duplicate cultures were subjected to each treatment condition. The cultures were grown for 7–10 days to reach confluency and subcultured at an inoculum of $1-5 \times 10^5$ cells per 75-cm² flask. Cells were maintained in continuous culture for up to 40 passages; periodically, 10^6 cells were tested for growth in soft agar¹⁸. Tumorigenicity determinations were carried out by subcutaneous injection of 3 day old non-immunosuppressed hamsters with $2-10 \times 10^6$ cells. Animals were checked weekly for the appearance of palpable tumours over a period of one year. Tumour development in positive animals generally required 2–3 months. All cell cultures which did not transform senesced within 20 passages.

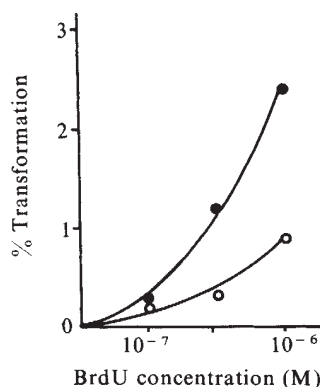


Fig. 3 Frequency of morphologically transformed colonies induced by treatment of BrdU plus near UV light. SHE cells were treated and scored for morphological transformation as described in the legend to Table 2. Cells were treated with the specified dose of BrdU for 24 h followed by irradiation with near UV for 3 min (○) or 5 min (●).

Cultures treated with BrdU plus near UV irradiation also exhibited neoplastic transformation (Table 3). In one experiment, transformation was observed for one of two cultures treated with BrdU (2.5×10^{-6} M) followed by one minute irradiation, as well as one of two BrdU treated cultures irradiated for three minutes. BrdU treated cultures subjected to higher doses of irradiation did not recover from the cytotoxicity of the treatment and senesced after one to two passages. In a second experiment, transformation occurred in both cultures treated with BrdU plus irradiation for three minutes, but was not observed in either culture treated with BrdU plus irradiation for one minute. Cells from all transformed cultures exhibited colony formation in soft agar between 20 and 30 passages after treatment (0.01–10% efficiency of colony formation) and formed tumours within three months after injection of 2×10^6 cells into newborn hamsters.

The observation that four of eight cultures treated with BrdU plus near UV irradiation transformed indicates that this treatment induces neoplastic transformation. The transformation of these cultures was detected earlier after treatment than the culture treated with near UV only, but later after treatment than benzo(a)pyrene-treated cultures, which transform 6–15 passages after treatment^{19,20}. It is not clear whether the transformation of the culture treated only with near UV light represented induced transformation or a spontaneous transformation event. Spontaneous neoplastic transformation has been observed in approximately 1 in 50 untreated cultures

carried out in our laboratory^{19,20}. It is important to study further the progression of neoplastic transformation induced by this direct perturbation to DNA.

The above experiments indicate that the BrdU plus near UV irradiation can induce cytotoxicity, DNA strand breakage, somatic mutation and neoplastic transformation of SHE cells. In contrast, BrdU treatment or near UV irradiation alone did not produce these effects. As the action of BrdU plus near UV irradiation is very specific, causing a direct perturbation of cellular DNA, our results suggest that such a direct perturbation is sufficient to initiate neoplastic transformation. This is strongly supported by recent experiments with synchronised cultures of SHE cells (T. T., J. C. B. and P. O. P. T., manuscript in preparation). Cells treated with BrdU plus near UV irradiation in the DNA synthetic period of the cell cycle but not those treated in the G_1 period or in late S/ G_2 phase were induced to undergo morphological and neoplastic transformation. These results strongly support a role of DNA damage in the initiation of transformation, and provide the first direct evidence that a DNA specific lesion(s) is sufficient to induce neoplastic transformation. Our study provides a valuable system for investigation of the molecular nature of the lesions leading to neoplastic transformation, as well as the role of DNA repair in the expression of this process.

We thank Brian Crawford for helpful discussions. This work was supported in part by grant 5P01 CA16043 and contract N01-CP-55713.

Received 10 February; accepted 15 May 1978.

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letters to nature

Neutrino stability and cosmological helium production

THERE is at least one lepton in addition to the electron and muon and this might have an associated neutrino. If such a neutrino is stable, its existence implies that the amount of helium produced in the early stages of the expanding Universe is higher than is generally believed. If there are several heavy leptons with stable neutrinos, the helium production may become so high that it is in clear contradiction with observations. It is pointed out here that, even if these neutrinos exist,

they may not be stable and that there is no clear evidence that even the muon neutrino is stable. If the additional neutrinos are unstable with a short enough half life, they do not affect the cosmological helium production and it is reduced if the muon neutrino is unstable.

Since the discovery of the cosmic microwave radiation¹, and the realisation that most astronomical objects whose chemical composition is known have a similar helium content², it has been generally accepted that most of the helium was produced in the very early stages of the Universe. It is also probable that D , ^3He and ^7Li have a similar origin. In the standard model of the Universe, the predicted helium production is in the

approximate range 23–27% by mass³, the precise value depending on the present mean density of the Universe.

Although most objects for which a reliable chemical composition is known have a helium content in excess of the minimum value of 23% quoted above, there are some suggestions of lower values. The most embarrassing ones relate to the Sun, where determinations of the helium abundance in the solar wind and solar cosmic rays (see ref. 4) have given lower values including one determination as low as 16%. The solar wind and solar cosmic ray abundances may not be typical of the solar photosphere and the lowest value is almost certainly incorrect but, as we cannot obtain a good direct measurement of the photospheric abundance, the possibility remains that the Sun does have a quantity of helium which is incompatible with the predictions of standard cosmological theory or at least very close to the lower limit allowed by that theory.

A recent discovery in elementary particle physics^{5,6} threatens to make the situation worse. It seems that a heavy lepton (τ) has been discovered, with a mass of about two proton masses. Although the properties of τ are uncertain, Ali and Yang⁷ suggest that like the electron and muon it may possess an associated neutrino ν_τ . This is also suggested by puzzling neutrino experiments reported by Miller⁸. If we suppose that ν_τ as well as ν_e and ν_μ is massless and stable, the introduction of ν_τ into the early Universe causes a more rapid expansion³. This means that there is less time for the conversion of neutrons into protons. When helium production occurs essentially all of the neutrons and an equal number of protons are converted into helium and this implies a greater helium production than in the standard model. If the time to reach a given temperature T is now t' instead of t in the standard model,

$$t'/t = (36/43)^{1/2} = 0.915$$

Greenstein⁹ calculated the effect on the helium production of a speed-up in the expansion of the Universe. Using his results with the above value of t'/t , the range of 23–27% quoted earlier becomes ~ 25 –29%. It is interesting that these are essentially the values given by the standard theory before account was taken of a reduction in the believed half-life of the neutron¹⁰. There is a further increase in the predicted helium production, if there are more heavy leptons with associated neutrinos. Although there is as yet no clear contradiction between theory and observation even if ν_τ exists, the discovery of additional leptons might easily lead to such a contradiction.

The ways of modifying the standard theory to produce less helium include filling the Universe with degenerate neutrinos¹¹, to force the conversion of neutrons to protons, or suggesting that the Universe was initially anisotropic and expanded so rapidly that there was a restricted time for helium production^{12,13}. Both of these are *ad hoc* suggestions and the free parameters which they introduce have to be chosen carefully to give the required helium production instead of no helium at all.

I suggest that there is still an uncertainty in elementary particle physics which might enable the standard theory to have a helium production lower than is at present predicted even if the ν_τ exists. As has already been mentioned, it is usually assumed that all neutrinos are massless and stable and that each type of massive lepton (and neutrino) has associated with it an absolutely conserved lepton number. The latter suggestion arises because the simplest decays of muons into electrons, for example, do not occur. It is, however, possible that some at least of the neutrinos might have finite mass and might be unstable. There would certainly be some attraction in having only one conserved lepton number, which would be possible if ν_μ and ν_τ (and any neutrinos associated with even heavier leptons) were to decay into some mixture of ν_e , $\bar{\nu}_e$ and possibly photons. Very simple decay schemes are ruled out by the failure to observe direct muon decay to electron.

Whether or not, neutrino instability affects the helium production depends on both the mass and the half life of the neutrino and on the precise properties of the weak interactions,

and we will not attempt to discuss all possible cases. At some stage in the expansion of the Universe neutrinos decouple from other matter in the sense that their mean free time between collisions becomes and remains longer than the age of the Universe. According to the charged current theory of weak interactions (ref. 14) ν_μ decouples shortly after the muon pairs decay ($t \sim 10^{-2}$ s), because the main interaction of ν_μ is with muons. The decoupling of ν_e occurs later ($t \geq 1$ s). The introduction of neutral currents^{15,16} means that there is, in fact, a direct interaction between ν_μ and $e^\pm$. As a result, although ν_μ will still decouple first, the time difference will be much less and will depend on the relative strength of the charged current and neutral current interactions.

We suppose that the masses of all neutrinos are sufficiently small that they behave like massless particles until after decoupling. Even if ν_μ , for example, is unstable ν_μ , $\bar{\nu}_\mu$ pairs will be present until they decouple but, if their half life is significantly less than the age of the Universe then, they will rapidly disappear. If their decay produces ν_e , $\bar{\nu}_e$ and γ and if there is sufficient time before ν_e also decouples, it may be possible for the ν_e and $\bar{\nu}_e$ to thermalise. If this happens the only particles affecting the immediate subsequent expansion of the Universe are $e^\pm$, γ , ν_e and $\bar{\nu}_e$ all in thermodynamic equilibrium and the expansion of the Universe is slower than if ν_μ are also present. Here we assume that what is true for ν_μ is also true for ν_τ and any other neutrinos. The result of this decay of ν_μ and ν_τ is to lead to a reduction in helium production to the approximate range 21–25%.

If, in contrast, ν_μ and ν_τ decay after all of the neutrinos have decoupled, this has no effect on the helium production, unless they decay almost immediately after ν_e decouples and unless they partly decay into photons. If their only decay products are ν_e and $\bar{\nu}_e$ these will continue to contribute to the expansion rate of the Universe. If some decay energy is turned into photons before the onset of helium production, these photons can thermalise and there will be some slowing down in the expansion rate and a related reduction in helium production.

I have deliberately concentrated on the effect of neutrinos on the ^4He abundance and not discussed D and ^3He . Their abundances depend critically on the mean density of the Universe³ and they are also increased by a more rapid expansion of the Universe⁹. A small increase in expansion speed leads to an increase in the mean density of the Universe implied by the observed abundances and this may give better agreement with other estimates of the density, such as that obtained from the smoothed out density of galactic matter. However, there is no such advantage if the ^4He abundance clearly contradicts with observations.

In conclusion, heavy leptons with associated neutrinos could lead to an increase in cosmological helium production and their discovery might make the standard theory conflict with observations. If some of the neutrinos are unstable with a short enough half life, and if the properties of the weak interactions allow electron neutrinos to decouple sufficiently long after neutrinos of other types, this problem may be avoided. If other evidence for cosmological helium production which is incompatible with the existence of more stable neutrinos seems sufficiently strong, this will suggest that, if additional neutrinos are discovered, they must be unstable.

I thank Michael Rowan-Robinson for helpful comments.

R. J. TAYLER

Astronomy Centre,
University of Sussex,
Falmer, Brighton, UK

Received 22 March; accepted 26 May 1978.

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Excess ^{15}N in the martian atmosphere and cosmic rays in the early Solar System

VIKING 1 and 2 have collected various data on the amount and composition of the martian atmosphere. The isotopic ratios $^{15}\text{N}/^{14}\text{N}$, $^{40}\text{Ar}/^{36}\text{Ar}$ and $^{129}\text{Xe}/^{132}\text{Xe}$ have been found to be distinctly different from the terrestrial values^{1–3}. We propose here that the enrichment of ^{15}N amounting to 62% (ref. 4) may be caused by nuclear reactions with intense cosmic rays in the early Solar System. The implications of the ratios $^{40}\text{Ar}/^{36}\text{Ar}$ and $^{129}\text{Xe}/^{132}\text{Xe}$ are different from that of $^{15}\text{N}/^{14}\text{N}$, because although ^{40}Ar and ^{129}Xe have a long-lived radioactive parent nuclide, ^{15}N does not. These two isotopic ratios may be the results of the initial endowment of the relevant elements, the degree of outgassing from the interior and recondensation or trapping in the surface region.

The tenuous atmosphere and the weak magnetic field of Mars, places Mars' cosmic ray irradiation effect on the surface somewhere between that of the Earth and the Moon. Even solar flare particles can irradiate the atmosphere because of low cutoff rigidities in the weak magnetic field and induce many nuclear reactions, but almost all of them will stop in the atmosphere and cannot penetrate the solid surface. Galactic cosmic rays can pass through the present thin atmosphere and also irradiate the surface rocks and soils, but should have been attenuated in the more dense atmosphere of early Mars. Therefore, the current atmosphere of Mars may preserve the integral cosmic ray irradiation effects which have been present since it acquired its atmosphere. Some excess may be expected for rare isotopes of volatile elements.

The excess ^{15}N may have been produced from the target nuclide ^{16}O , which constitutes the major atmospheric component as CO_2 , by cosmic ray irradiation. The amounts of the target nuclide ^{16}O and the excess ^{15}N in the present atmosphere are calculated from the Viking observations^{5,6} as $\sim 5 \times 10^{23} \text{ atom cm}^{-2}$ and $\sim 3.5 \times 10^{19} \text{ atom cm}^{-2}$, respectively. Here, the initial ratio of $^{15}\text{N}/^{14}\text{N}$ is assumed to be the same as the terrestrial value of 0.00368.

We estimate a necessary cosmic ray flux required to explain the excess ^{15}N , assuming the amount and the chemical composition of the early atmosphere as follows. The amount of the early martian atmosphere was estimated^{2,7} as ~ 250 – $\sim 1,300 \text{ g cm}^{-2}$ from the present atmosphere⁶ of $\sim 19 \text{ g cm}^{-2}$, depending on models of the initial endowment of volatiles and the degree of outgassing. We adopt a value of 300 g cm^{-2} , taking into account the above values and for the reason given below. The early atmosphere may have decreased in density to the present condition as a result of the deposition of CO_2 as carbonates⁷. The possible deposition of N_2 as nitrates or nitrites should have had a small role in decreasing the amount of the atmosphere, as CO_2 has probably always been the most abundant gas. The composition of the early atmosphere is assumed to be same as the present atmosphere⁵. Then the amount of ^{16}O and the excess ^{15}N in the early atmosphere are evaluated as $\sim 8.2 \times 10^{24} \text{ atom cm}^{-2}$ and $\sim 5.7 \times 10^{20} \text{ atom cm}^{-2}$, respectively. The effective cross section of the reaction $^{16}\text{O}(\text{p}, \text{x})^{15}\text{N}$ is estimated as $\sim 100 \text{ mbarn}$ from the measured cross section⁸ of $^{16}\text{O}(\text{p}, \text{pn})^{15}\text{O}$. From these values, the required integral cosmic ray flux is calculated as $6.9 \times 10^{20} \text{ proton cm}^{-2}$ ($E > 30 \text{ MeV}$).

Isotopic anomalies of other elements may be predicted as follows. ^{17}O and ^{18}O can be produced from CO_2 by the reaction $^{16}\text{O}(\alpha, \text{p}2\text{n})^{17}\text{F}(\beta^+)^{17}\text{O}$ and $^{16}\text{O}(\alpha, \text{pn})^{18}\text{F}(\beta^+)^{18}\text{O}$ with α particles of $6.9 \times 10^{19} \text{ particles cm}^{-2}$ assuming α/p ratio as 1/10. The effective cross section of (α, pn) is estimated as $\sim 200 \text{ mbarn}$ from the measured values⁹. The effective cross section of $(\alpha, \text{p}2\text{n})$ is assumed to be the same as that of (α, pn) . The expected anomalies of $^{17}\text{O}/^{16}\text{O}$ and $^{18}\text{O}/^{16}\text{O}$ are $\sim 4\%$ and $\sim 0.7\%$, respectively, assuming that the initial isotopic composition of oxygen is the same as the terrestrial value. These expected anomalies are within the experimental accuracies^{1,2,4,5}. Neon is another candidate of isotopic anomalies, although only the abundance of ^{22}Ne was determined by the Viking missions⁵. Neon is produced in only negligible amounts in the atmosphere because of the lack of abundant target nuclides, but Ne can be produced in the solid surface by secondary cosmic rays. However, the amount of cosmogenic Ne in the present atmosphere may not be so large, because the observed abundances of rare gases show a 'planetary pattern' which is common to chondrites and Earth⁵. If the amount of the early atmosphere is more than $\sim 300 \text{ g cm}^{-2}$, ^{22}Ne of less than $\sim 4 \times 10^{17} \text{ atom cm}^{-2}$ may be produced in the solid surface¹⁰. There is no contradiction with the observed abundance⁵ of ^{22}Ne of $\sim 6 \times 10^{16} \text{ atom cm}^{-2}$ if less than $\sim 10\%$ of the produced ^{22}Ne has been released into the atmosphere. In this case, a considerable excess of ^{21}Ne is expected.

In discussing a possible cause of the intense cosmic rays, Strong¹¹ suggested that the two of the 11 unidentified γ -ray sources reported by the COSB group¹², CG135+1 and CG189+1, can be identified as W3 and NGC2175, respectively. These objects are star associations in a very early evolutionary stage. The estimated cosmic ray density in W3 is $10^4 \times$ 'the local density'. The Solar System should have been born in a star cluster like W3. It may be plausible that the cosmic ray density in the cluster in its early history had been as high as in W3, because the particles could either have been accelerated in the stars or in associated supernovae or supernovae remnants in the cluster. If the cosmic ray density in the cluster had been $10^4 \times$ 'the local density', the required flux of $\sim 6.9 \times 10^{20} \text{ proton cm}^{-2}$ could have been attained within a time span of $\sim 3 \times 10^7 \text{ yr}$. The life time or the disruption time of a cluster is estimated¹³ as $2.8 \times 10^8 \rho \text{ yr}$ where ρ is density in $M_\odot \text{ pc}^{-3}$. We do not know when the atmosphere of Mars was formed, although Masursky *et al.*¹⁴ suggested from the study of fluvial channels that the dense atmosphere permitting liquid water to flow had been formed at least $3.5 \times 10^9 \text{ yr BP}$. If the martian atmosphere had formed within $\sim 10^8 \text{ yr}$ after the formation of Mars (the same time for the Earth's atmosphere¹⁵) the cessation time of the high cosmic ray density, $\sim 1.3 \times 10^8 \text{ yr}$ after the birth of Mars, would be reasonable compared with the life time of clusters shown above.

The Sun might also be a possible source of the intense cosmic rays if the early Sun could produce cosmic rays with an energy spectrum like that from galactic cosmic rays.

Do the above arguments conflict with our knowledge of the history of cosmic rays? It is generally accepted that the average intensity of galactic cosmic rays has been constant¹⁶ within a factor of two over the last $\sim 10^9 \text{ yr}$. But unfortunately, we do not have any knowledge of cosmic rays beyond $\sim 2 \times 10^9 \text{ yr BP}$ (ref. 16). The martian atmosphere may be one of the invaluable samples of the cosmic ray history of this era.

We thank Drs S. Tanaka, M. Honda, K. Sakurai, M. Ozima, H. Mabuchi, K. Yamakoshi and T. Shimamura for useful discussions.

SHOHEI YANAGITA

Cosmic Ray Laboratory,
University of Tokyo,

MINEO IMAMURA

Institute for Nuclear Study,
University of Tokyo,
Tanashi, Tokyo 188, Japan

Received 3 March; accepted 17 May 1978.

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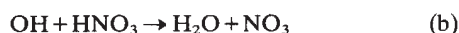
Ratio of HNO₃ to NO₂ concentrations in daytime stratosphere

A RECENT NASA report¹ discussed the relative amounts of nitric acid and nitrogen dioxide present in the stratosphere, and pointed out that the values of the ratio [HNO₃]/[NO₂] measured by Evans *et al.*² are, in fact, considerably lower than the values predicted theoretically using the latest values for rate constants and photodissociation coefficients for the appropriate reactions. This letter reports independent determinations of the [HNO₃]/[NO₂] ratio which apparently support the measured values² at levels above 25 km, but which are nearer the calculated values below this level.

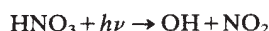
The ratio of [HNO₃] to [NO₂] is determined¹, in conditions of photochemical equilibrium (that is, with no transport) by the relationship

$$\frac{[\text{HNO}_3]}{[\text{NO}_2]} = \frac{k_a \cdot [\text{OH}] \cdot [\text{M}]}{J_{\text{HNO}_3} + k_b \cdot [\text{OH}]} \quad (1)$$

where k_x denotes the reaction rate for reaction $x = a$ or b , where



and where J_{HNO_3} denotes the photodissociation coefficient for the reaction



The ratio depends on the concentration of the hydroxyl radical present, [OH], and it has been suggested¹ that the good agreement which Evans *et al.* found between their measurements and their one-dimensional model calculations was a result of a fortuitous calculated value of [OH]. More recent reaction rates lead to disagreement between theory and experiment¹. Simultaneous sub-millimetre spectroscopic studies of HNO₃, NO₂ and H₂O have provided experimental data³ to help resolve the question of the [HNO₃]/[NO₂] balance. These results, which were taken during local noon on 24 September 1974 at a latitude of 44°N, have been re-analysed here to provide values of this ratio. The methods used to measure the concentrations are described in ref. 3.

The results of this re-analysis are presented in Fig. 1. The values of the ratio [HNO₃]/[NO₂] are found to range monotonically from ~3.6 at 23 km to ~0.18 at 32 km. Also shown are the results of Evans *et al.*² and those of several one-dimensional model calculations¹ (the shaded area represents the range of results obtained with different models rather than any assessment of uncertainty). Between ~25 and 33 km, the agreement between our values and those of Evans is very good, but below 25 km our results lie closer to the theoretical values.

In a detailed comparison of the two sets of data it must be remembered that the NPL results were obtained during local noon. The Canadian results², however, were obtained at a later local time: the HNO₃ data were obtained in late afternoon at

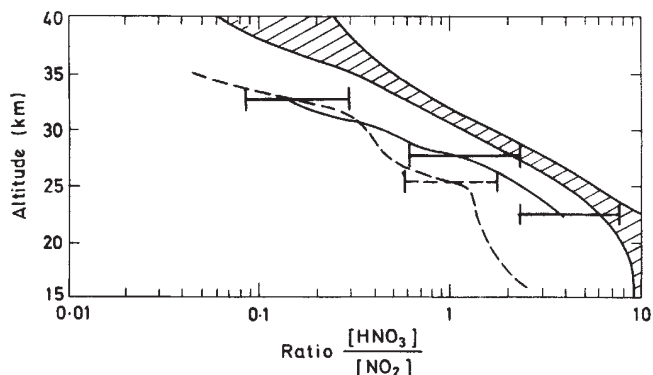


Fig. 1 Values of the ratio [HNO₃]/[NO₂] derived from the NPL results³ (solid line and solid error bars), compared with similar data due to Evans *et al.*² (broken line and broken error bar), and with the results of theoretical calculations using one-dimensional models¹ (hatched area).

solar zenith angles of 75° to 84°, and the NO₂ data during the subsequent sunset, that is at solar zenith angles of 90° plus. The main concern might be the effects on the ratio [HNO₃]/[NO₂] due to diurnal variabilities, but relatively little change occurs in [NO₂] or [HNO₃] between midday and the onset of sunset, although the amount of NO₂ can, of course, increase by ~two-fold during a sunset due to reformation of NO₂ from NO (ref. 4). Thus the time difference for the Canadian NO₂ measurements² could affect the comparison with theory and with the present results, although without further information on the exact time relative to sunset of the NO₂ measurements, we cannot conclude what that effect might be. This difficulty does not exist, of course, for the results reported here.

Uncertainties in our [HNO₃] and [NO₂] data were ± 25% and ± 35% respectively¹, so that the uncertainties in the ratio lie between +90% and -45%. Similar uncertainties are quoted by Evans *et al.*² and must be borne in mind when making the comparisons between the experimental and theoretical results. However, given the close similarity of our experimental results with those of Evans *et al.*, at least above 25 km, we conclude that these new results confirm a significant disagreement between the results from one-dimensional atmospheric models and measured values for the profile of the [HNO₃]/[NO₂] ratio above this level. Below 25 km the new results lie close to the calculated values. The full significance of the differences between observations and theory cannot, however, be fully evaluated without a clearer idea of the uncertainties in the results of the model calculations. Clearly, simultaneous measurements of HNO₃, NO₂ and OH will be required to fully test the model predictions.

J. E. HARRIES

National Physical Laboratory,
Teddington, Middlesex, UK

Received 29 March; accepted 9 June 1978.

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Uniform distribution of BeO particles in Be casting produced in rocket free fall

CAST beryllium is coarse-grained and, consequently, brittle. Much hot working is required to refine the grain structure of a cast beryllium ingot before good ductility is developed. Attempts at grain refining with for example, ultrasonics in the melt and stirring of the melt, have been unsuccessful. Research

has also been undertaken (unsuccessfully) to find a grain-refining agent suitable for beryllium¹. A good grain refiner must be retained in the melt in the form of fine, dispersed particles. Particles in molten beryllium, however, because of its very low density, agglomerate and segregate from the melt terrestrially. This is believed to be due primarily to Stokes collisions and velocity gradient collisions. As the Stokes velocity is directly proportional to the acceleration due to gravity, the rate of agglomeration and subsequent segregation from the melt ought to be reduced considerably in the microgravity environment of space. We report here the results of an experiment in the weightless environment of space, using a sounding rocket equipped with the NASA electromagnetic containerless processing payload (ECP), to melt and solidify a specimen of beryllium containing beryllia, an inoculant tried previously as a grain refiner.

The difference in density between the particles and the molten beryllium causes particles to settle downwards in a melt. In a quiescent melt, the particles settle to the bottom of the melt. The rate of settling of these particles (assumed spherical) is proportional to the square of the radius. Large particles settle faster than smaller particles; in settling they collect the smaller particles and form agglomerates—these are called Stokes collisions². In a non-quiescent melt with electromagnetic stirring and convection (gravity driven and surface tension driven) present, the particles move in complex orbits. Velocity gradients sweep particles together—these are called velocity gradient collisions^{2,3}. In a non-quiescent melt, simple settling out of the dispersant is not observed. Superimposed on the particle motion caused by fluid motion is the differential Stokes settling. This is believed to cause the observed agglomeration and separation from the melt, which should be reduced in the environment of space because the differential Stokes settling due to gravity is eliminated in the microgravity environment.

Reduction in the rate of agglomeration and separation of dispersants from melts has been previously observed in materials processing in space experiments carried out on Skylab and SPAR (space processing application rockets) sounding rocket experiments. In Skylab certain alloy compositions immiscible on Earth were produced as a dispersion⁴, whisker reinforced composites not available on Earth were produced⁵ and in SPAR I thoria particles were dispersed in magnesium more uniformly than on Earth⁶.

An experiment was therefore flown in the microgravity⁷ environment of space, using a sounding rocket (SPAR 3) equipped with the NASA (ECP)^{7,8}, to melt and solidify a specimen of beryllium containing beryllia, an inoculant thought to be a possible grain refiner for beryllium. The beryllium melted and solidified was Kawecki-Beryllco Industries HIP-50 which is a high purity hot isostatically pressed (from powders) beryllium grade containing 1.5% BeO by weight.

The flight experiment involved melting and solidifying a 0.922 cm spheroid of HIP-50 in the ECP during the 230 s of weightlessness obtained in the 'free fall portion' of the SPAR sounding rocket flight. The atmosphere was 800 torr of research grade argon. The specimen was positioned, heated and melted within a cusp coil by the electromagnetic field of the coil. The sequence of operation during flight consisted basically of: (1) a high power mode wherein the specimen was heated and (2), a low power mode which the specimen was allowed to solidify with only enough applied power to keep it from contacting the coil so that stirring was kept to a minimum.

Experiments on Earth were carried out in a 'breadboard facility,' which duplicated the ECP facility in all essential aspects so that the major difference between the ground based (GB) experiments and the flight experiment was the effect of gravity. The materials used for both the GB experiment and the flight experiment were from the same billet, so that there were no differences due to pressing or powder lot variables.

The distribution of BeO particles was studied in detail by scanning electron microscopy (SEM). These SEM photos were

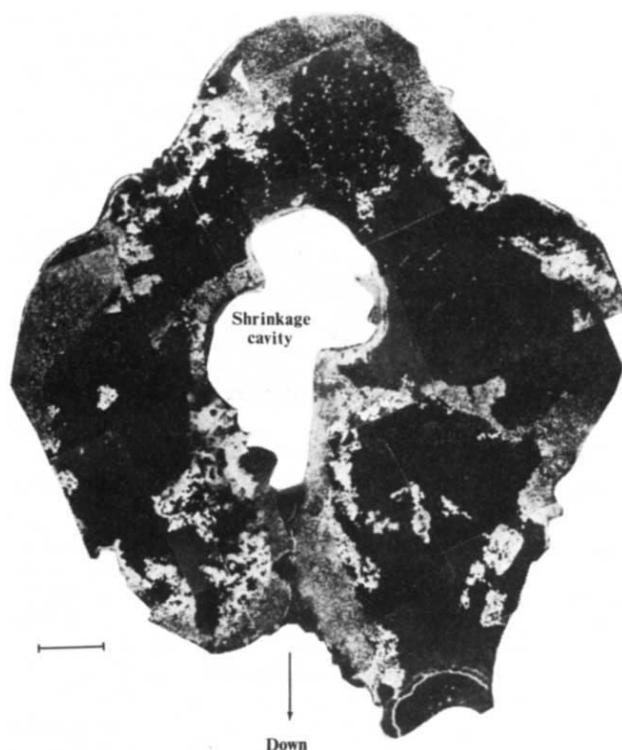


Fig. 1 SEM micrographs of ground-based reference specimen no. 1. White regions are heavily agglomerated; dark regions are relatively free of oxide. Outline differentiates specimen from mounting material and shows shrinkage cavity (in centre). Scale bar, 0.1 cm.

taken on sections polished so that the BeO particles stand out in relief above the Be matrix and are thus readily seen in the SEM⁹. Figure 1 is a montage of low power SEM micrographs of one of the GB specimens. Gross agglomeration and segregation of oxide from the melt evidently occurred. Electromagnetic stirring kept the BeO particles from settling to the bottom. In samples heated in conventional furnaces the BeO did settle to the bottom. The SEM micrographs of the flight specimen (Fig. 2) show that the distribution of oxide particles is much more uniform in the space experiment. There is a darker band evident in the flight specimen which may arise from a vortex line. All regions of the flight specimen showed an oxide dispersion in contrast to the GB specimen which showed a heterogeneous microstructure containing heavily agglomerated regions and regions relatively free of oxide.

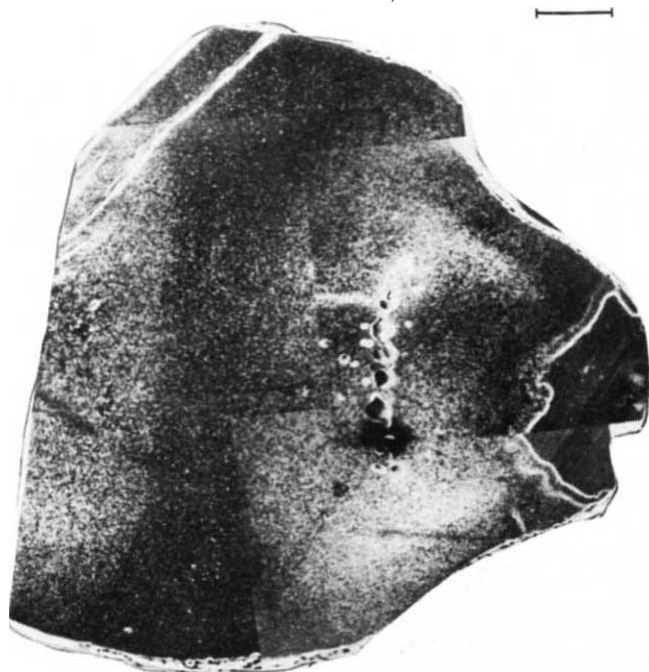
Detailed SEM studies (as in ref. 8, and carried out by V. Damiano) at magnifications of up to $\times 30,000$ further confirmed the more uniform nature of the BeO distribution in the flight specimen. The range of BeO particle sizes observed is from 0.2 to 1.3 μm in the original material, the GB specimens and the flight specimen. The difference is in the state of agglomeration after melting and solidification. Very detailed Quantimet analysis^{8,9} using many SEM photos at $\times 1,000$ and $\times 3,000$ magnification showed the volume percentage of oxide varied from 0.0005 to 2% in GB samples and from 0.1 to 2% in the flight sample. The average value in the starting material was ~ 1 vol. %.

The objective of obtaining a more uniform, unagglomerated oxide dispersion by melting and solidifying in the microgravity environment of space was apparently met. To our knowledge, beryllium with an unagglomerated dispersion of oxide has not been prepared by casting before. We suggest that the reduction of particle collisions, Stokes and velocity gradient, resulted in lengthening the agglomeration time considerably.

The wetting or non-wetting of the dispersed particles by the melt will affect the times of agglomeration and separation from

the melt. The tendency to agglomerate will be greater for particles which are not wetted by the melt. Wetting is defined¹⁰ by the contact angle between the solid and liquid at the boundary line between the three interfaces: solid-vapour, liquid-vapour, liquid-solid. An example of this is a droplet of the liquid resting on a solid surface. When the contact angle is less than 90°, the liquid wets the solid. Molten beryllium does not wet BeO because the contact angle exceeds 90°. Hence, there is an additional driving force for agglomeration and separation from the melt, as the lower energy configuration is that which presents a minimum surface of BeO to the molten beryllium.¹⁰ The importance of wetting to agglomeration phenomena in dispersions and to bonding in metal-ceramic composites (whisker composites) has been previously discussed¹¹⁻¹³. It is believed that the results of this experiment indicate that the gravity-driven agglomeration mechanisms, that is, settling and gravity-driven convection, are important mechanisms for BeO particles dispersed in molten beryllium. Other research²⁻⁶ supports this conclusion for other dispersions. The microgravity environment does not eliminate agglomeration and separation. It seems to lengthen the time for appreciable agglomeration to occur. The contribution of other mechanisms, such as electromagnetic stirring, surface-tension driven convection, and driving forces due to non-wetting of the dispersant by the melt may be more accessible to study in the microgravity environment, in which they would be the controlling factors in agglomeration. Hence, experiments may be devised to vary these factors independently in the microgravity environment, with greatly reduced interference by gravity-driven mechanisms.

Fig. 2 SEM micrographs of flight specimen. Cutting facet is seen at right and indications of small part of shrinkage cavity are seen near centre. Scale bar, 0.1 cm.



The grain size of both the GB specimens and the flight specimen was large (>150 μm), thus, a fine dispersion of oxide in the melt did not result in grain refinement. One hypothesis is that the BeO particles were spheroidised (grain refining agents should be angular and sharp-cornered) either during the hot isostatic pressing or during the time while molten. Further research should use a starting material prepared so as to contain unspheroidised particles and the time while molten should be decreased to test the hypothesis that BeO is a suitable grain-refining agent for beryllium.

The results of this rocket experiment (and the Skylab and SPAR I flights) indicate that space experiments offer a unique

situation for certain metallurgical systems where uniform distribution of particles cannot be produced on Earth. In these systems uniform distribution of particles can be used for the first time to test for grain refining agent efficacy in casting, possible dispersion hardening effects, and so on.

This work was funded by NASA at the Marshall Space Flight Center on contract NAS8-31963, which was monitored by Fred Reeves.

G. WOUCH

*General Electric Space Sciences Laboratory,
and Department of Materials Science,
Drexel University, Philadelphia, Pennsylvania 19104*

R. T. FROST

*General Electric Space Sciences Laboratory,
King of Prussia,
Pennsylvania 19101*

N. P. PINTO

G. H. KEITH

*Kawecki Berylco Industries, Inc.,
Reading, Pennsylvania 19603*

A. E. LORD JR

*Department of Physics and Atmospheric Science,
Drexel University, Philadelphia, Pennsylvania 19104*

Received 3 February; accepted 23 May 1978.

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Spinodal decomposition in naturally occurring non-cubic spinels

TRANSMISSION electron microscopy (TEM) of non-cubic, naturally occurring chrome-rich magnesioferrite spinels has revealed the presence of a fine lamellar structure which is shown here to account for the complex X-ray diffraction patterns recorded previously¹. These submicroscopic lamellae are interpreted as having been formed by oxidation spinodal decomposition of iron-rich chromites and indicate the presence of an immiscibility region previously not recognised.

X-ray diffraction studies of anisotropic chromian spinel from Rødøya, Norway, indicated that this mineral was non-cubic¹, and had close similarities to donathite². More detailed studies³ revealed slight lattice distortions which were shown to be orthorhombic. Some material from Rødøya has been ground in an agate mortar before examination by a Philips EM300 transmission electron microscope equipped for high resolution imaging. A second sample was mechanically polished to approximately 50 μm thickness and finally thinned by argon ion bombardment with a glancing angle of 15°. The thinned and crushed samples give identical results.

TEM has shown that the spinel contains numerous mutually perpendicular narrow lamellae (maximum width ~50 nm) approximately parallel to {100} (all symbols of this type refer to pseudocubic axes). Exsolution along mutually perpendicular directions in a cubic, or near-cubic, mineral is common and our

work has been concentrated on those regions having unidirectional lamellae. These have sharp interfaces (Fig. 1) and within the light lamellae there is a 6–10 nm platelet substructure (S). These features are identical to those which have been recognised in silicate minerals^{4,5} but their presence in spinels is demonstrated here for the first time. By analogy with work done in experimental oxide and element systems the lamellae are considered to have been produced by spinodal decomposition. The difference in diffraction intensity between domains is probably associated with chemical differences which cause a variation in the structure scattering amplitude⁶ because the intensity variations as a result of crystal orientation are likely to be small.

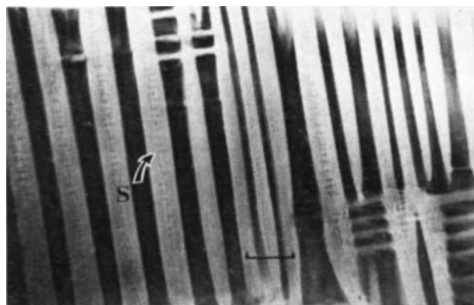


Fig. 1 Transmission electron micrograph of ion-thinned Rødøya spinel illustrating the three phase composition of lamellae and platelet substructure (S). Scale bar, 250 nm.

Anisotropic chromian spinels for which cubic symmetry was deduced on the basis of X-ray diffraction data were examined in the same way and these, together with isotropic cubic chromites, were homogeneous. An exception was a sample from Værness¹ where extremely fine and poorly developed lamellae were observed. Re-examination of the X-ray data shows that there is a tendency for broadening of the (440) peak¹. There seems to be a correlation between composition, in terms of iron content in particular, and development of lamellae in the Norwegian spinels. To test this a sample of similar composition was obtained from the Chimwadzulu Hill peridotite, Malawi⁷, and in this identical lamellae structure was observed.

High resolution TEM images were generated to determine the crystallographic relationships of the lamellae to each other. Figure 2, recorded using the sample from Rødøya and with the electron beam approximately parallel to $[10\bar{1}]$, depicts a lamella (marked L) which at lower magnification is lighter than its neighbours (Fig. 1). A direct measurement of the interplanar angle $(111) \phi (1\bar{1}1)$ indicates that the area shown in Fig. 2 has a tetragonal unit cell. However, in the lamella L, $\phi = 70.3^\circ$ (that is, c is 0.830 nm when a is set at 0.836 nm based on X-ray diffraction data) and the neighbouring darker lamellae have

$\phi = 71.2^\circ$ (that is, c is 0.846 nm with a set at 0.836 nm). These values of c closely agree with cell dimensions reported earlier on the basis of single crystal diffractometry¹. They indicate the presence of both a contracted-tetragonal (light lamellae) and expanded-tetragonal (dark lamellae) phases in approximately equal amounts.

Other high-resolution imaging indicates the presence of small orthorhombic regions in the same Rødøya spinel². Figure 3 shows a four-beam lattice image recorded using the (220) , $(13\bar{1})$ and $(1\bar{1}1)$ reflections and the beam incident along $[112]$. In the region surrounding a, misalignment of (220) planes occurs and the interplanar angle $(220) \phi (1\bar{1}1)$, which is 90° in the region around e, is 86.5° while $d_{220} = 0.293$ nm, $d_{131} = 0.253$ nm and $d_{111} = 0.481$ nm. Relative measurements of the magnitudes of a and b can be made by counting the number of fringes crossing (100) and (010) axes and this results in a unit cell dimension for the area around a such that a or $b = 0.778$ nm and a or $b = 0.894$ nm. Such results are consistent with an orthorhombic inclusion in a tetragonal matrix. The area of distortion is small (~ 7 nm) and it seems likely that we have here imaged a platelet in a single lamella. The model therefore emerges of orthorhombic platelets occurring in contracted-tetragonal lamellae which are probably enriched in lighter elements (such as Mg and Al) relative to the expanded-tetragonal lamellae which may be enriched in heavier elements (Fe, Cr). More detailed modelling depends on comprehensive electron microscopy and computer simulation of distorted spinel structures and such work is in progress.

There is evidence for the presence of a low temperature solvus in naturally occurring low magnesium spinels of the chromite (cr)–hercynite (hc)–magnetite (mt) series similar to that which has been established for ulvöspinel, magnetite and pleonaste⁸. Purvis *et al.*⁹ have shown that low magnesium, chromiferous spinels in mafic rocks of the Carr Boyd Rocks Complex, Australia, commonly carry exsolution bodies of chrome pleonaste and/or ilmenite. The chrome pleonaste may occur as lamellae parallel to $\{100\}$ or $\{111\}$ of the host spinel. Muir and Naldrett¹⁰ suggested that exsolution caused the development of inhomogeneous spinel grains from originally single phase magmatic oxide crystals. Electron microprobe analyses of low magnesium chromian spinels from ultramafic rocks at Gian Nickel Mine, Canada, led them to infer a solvus in the cr–hc–mt system which projects from the hc–mt join and terminates at about 15 mole% chromite. This is incompatible with Cremer's¹¹ theoretical solvus for the same system (magnesium free) constructed from experimentally determined miscibility gaps. Berg (ref. 12 and personal communication) has shown that for Cr–Mg-spinels in ultramafic rocks affected by contact metamorphism there exists a solvus between Cr–Mg–hc and Cr–Al–mt which is compatible with that identified by Purvis *et al.*⁹ and by Muir and Naldrett¹⁰. Furthermore, in the grains of Cr–Al–mt there is a 'woven texture' or 'cloth-like fabric' caused by fine exsolution of an unidentified phase⁸.

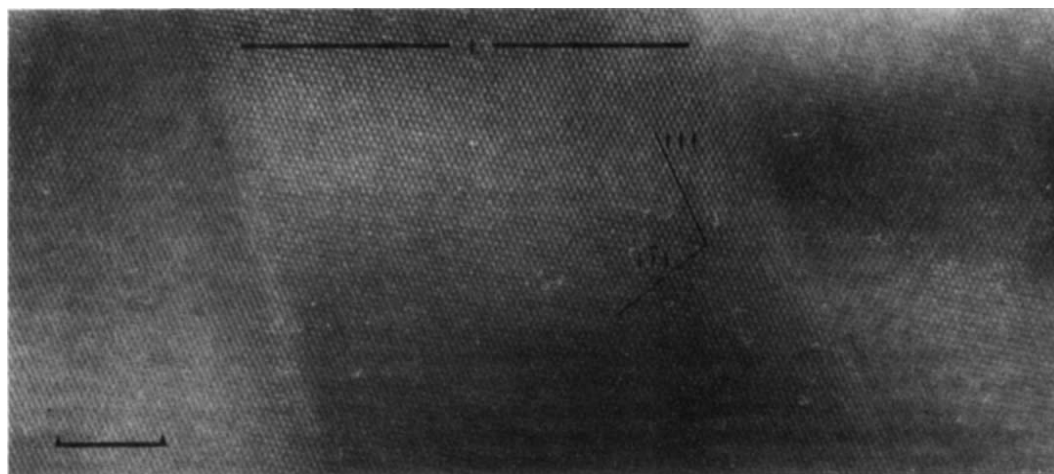


Fig. 2 High resolution lattice image of fragmented Rødøya spinel with the electron beam parallel to $[10\bar{1}]$. Central light lamella (L) flanked by two darker lamellae. Scale bar, 10 nm.

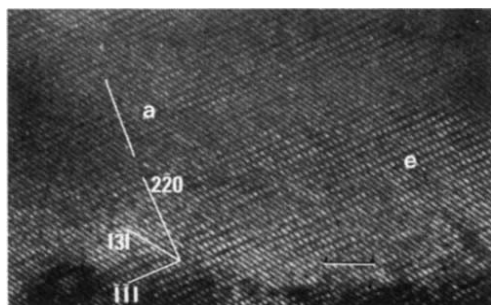


Fig. 3 High resolution lattice image of fragmented Rødøya spinel with electron beam parallel to $[112]$. Misorientation of (220) lattice planes in the region near a indicate an orthorhombic platelet. The lattice displacement is best seen by viewing the picture at a glancing angle along the white lines. Scale bar, 2.5 nm.

X-ray diffraction data have not been reported for any of the above samples.

On the basis of our work on the spinels from Rødøya, Værness and Chimwadzulu Hill we suggest that spinodal decomposition of iron-rich members of the spinel series can take place in addition to the chemical solvus between hercynite and magnetite established by other workers. The textures described by Muir and Naldrett¹⁰ probably indicate this phenomenon on a coarser scale. Our work has indicated that this spinodal decomposition can occur on a submicroscopic scale and leads to the development of distorted spinel structures which are predominantly tetragonal. The close similarity of the spinels from Rødøya and donathite^{1,2} strongly suggest that the latter mineral has a similar complex structure.

This work was supported by grants from the CSIR (S. Africa) and Norges Teknisk-naturvitenskapelige Forskningsråd (ACM) and University of Cape Town Staff Research Grants. We thank Dr Haslam for supplying material from Malawi.

ALAN C. MOORE

Department of Geology,
University of Cape Town,
Rondebosch, South Africa

DAVID CRAWFORD

Electron Microscope Unit
University of Cape Town,
Rondebosch, South Africa

Received 10 April; accepted 17 May 1978.

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Ultrastructure of calcite decomposition *in vacuo*

CALCIUM carbonate decomposes on heating to form calcium oxide—the quicklime or unslaked lime of industry. Because of its commercial significance any information concerning the nature of the CaO phase is important. I report here on the ultrastructure and crystallography of the reaction products obtained by the *in situ* and *in vacuo* thermal decomposition of calcite in the transmission electron microscope (TEM).

Thin rhombs of calcite were obtained by random cleavage of specimens of Iceland spar calcite. The rhombs were then prepared and mounted for ion-beam thinning parallel to the $(10\bar{1}4)$ cleavage faces following the procedure of Towe and Thompson¹. The ion-thinned wafers were vacuum coated with evaporated carbon to prevent charging and studied at 100 kV in a Philips EM-200 TEM. Appropriate regions were chosen for study and the mineralogy confirmed by selected area electron diffraction. The *in situ* thermal decomposition of the calcite was empirically accomplished by increasing beam diameter and beam current and/or removing condensor apertures as necessary. In this way the extent of decarbonation of the calcite rhomb-wafers could be monitored visually in the electron microscope and the nature of the phases involved confirmed through electron diffraction.

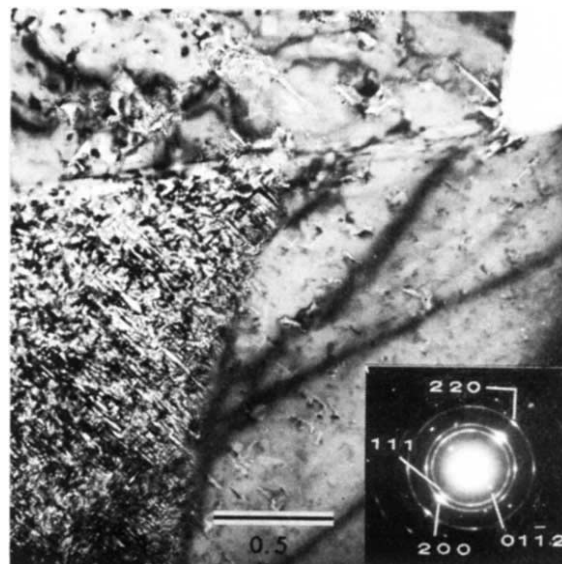


Fig. 1 Partially decomposed calcite crystal thermally altered *in situ* in the TEM. Inset: electron diffraction pattern from the area shown. (Scale bar, 0.5 μm .)

The most striking feature of the *in vacuo* decomposition of calcite as observed in the TEM is the very fine crystallite size of the CaO reaction product. A single crystal of calcite becomes a porous polycrystalline network of CaO needles, usually with evidence of preferred orientation. Figure 1 shows portions of a cleavage fragment resting on a $(10\bar{1}4)$ face. Some areas subjected to beam heating have decomposed to CaO while others remain as CaCO_3 . A higher magnification view (Fig. 2) shows the matted network of CaO crystallites and the inter-

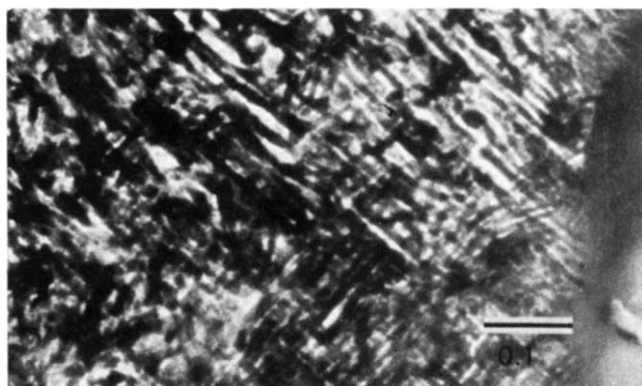


Fig. 2 Enlargement of a portion of Fig. 1 to show the needles of calcium oxide and the intercrystalline porosity. (Scale bar, 0.1 μm .)

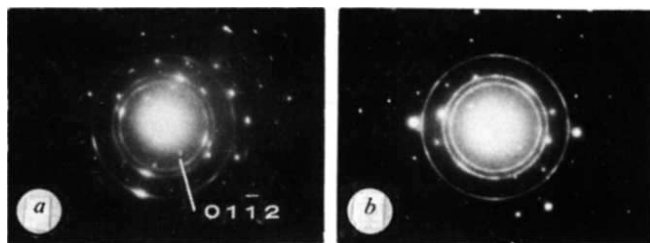


Fig. 3 Electron diffraction patterns of other regions of CaCO_3 decomposition. Note the lower degree of arcing of the rings in (b).

crystalline pores. Measurement of the crystallite widths reveals that most of the CaO needles are approximately $100 \text{ \AA}$ wide. The lengths are more difficult to measure but some are at least $0.1 \mu\text{m}$ long.

Figures 1 and 2 show several preferred orientations of the CaO needles. Electron diffraction patterns (Fig. 1, inset) typically show arcuate diffraction maxima from the CaO phase superimposed on the sharp spots of the remaining undecomposed calcite crystal. The most prominent of these fibre reflections are the (200), (220), and (111) of CaO normal to (01 $\bar{1}$ 2) of calcite. Other regions of decomposition show different preferred orientations (Fig. 3a) and in some patterns the more uniform nature of the rings (Fig. 3b) suggests a more random array of CaO crystallites. The evidence seems to indicate that several topotactic transformations are possible. This may account for some of the existing disagreement on this point²⁻⁴.

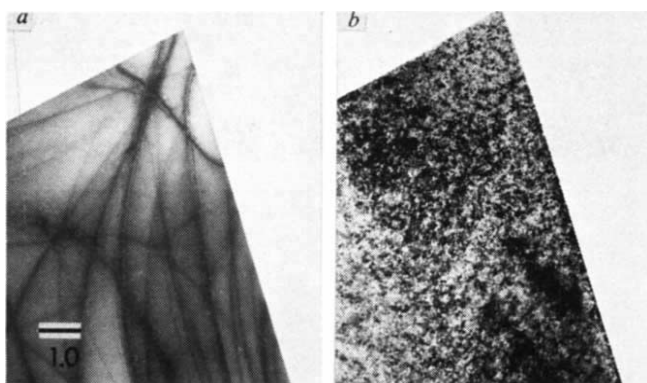


Fig. 4 A corner of a calcite rhomb (a) before, and (b) after decomposition. Complete transformation to CaO (b) makes little change in the overall crystal morphology. (Scale bar, $1.0 \mu\text{m}$.)

Beruto and Searcy^{5,6} have studied the decomposition of calcite both in air and nitrogen and *in vacuo*. They observed that the CaO produced *in vacuo* was more reactive to hydration than that produced in air or nitrogen, regardless of its crystallinity. Their molar volume calculations indicated that *in vacuo* decomposition produced particles with a high porosity (in excess of 50% porosity) but they were unable to resolve any pores in the scanning electron microscope⁶. As shown here the sharp cleavage edges of the original calcite (Fig. 4a) are minimally altered after thermal decomposition *in vacuo* (Fig. 4b). With a crystallite size near $100 \text{ \AA}$ the true nature of the CaO phase will not be easily resolved in the SEM.

The present TEM work confirms that the altered calcite crystals are indeed porous with the CaO in a very finely divided form. This combination of very fine crystallite size and high porosity explains the high reactivity of this material. Such porous material, even with an improved crystallite size (crystallinity), would still be expected to be more reactive than the comparatively coarse, rounded and sintered oxide⁶ produced by decomposition of calcite in air or in nitrogen. Electron

diffraction also confirms that the fine needles of CaO shown here are crystallographically identical to CaO formed in air. Any metastability that such calcium oxide might have when formed in large calcite crystals must then be due to its size and shape rather than to some basic structural difference⁵.

I thank D. E. Appleman for helpful discussion.

KENNETH M. TOWE

Department of Paleobiology,
Smithsonian Institution,
Washington, D.C. 20560

Received 3 May; accepted 19 May 1978.

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Mid-proterozoic sulphate evaporites at Mount Isa mine, Queensland, Australia

PSEUDOMORPHED sulphate evaporites have been discovered in the Middle Proterozoic (1,600–1,500 Myr BP¹) silica-dolomite sequence^{2,3} at the Mount Isa Mine, Queensland, Australia (20°44' S, 139°29' E). The silica-dolomites are host to the copper mineralisation at the Mount Isa mine^{2,3} and interdigitate with the fine-grained silver-lead-zinc-bearing pyritic and dolomitic Urquhart Shales of the Mount Isa Group¹. A close association of evaporites with copper^{4,5} and lead-zinc⁶ has been reported for many ore deposits. The discovery of replaced sulphate evaporites at Mount Isa reported here raises the possibility that this large ore deposit may have been formed from metal-rich brines of the kind forming today in evaporitic basins⁷ rather than from volcanically derived hydrothermal solutions^{8,9}.

The silica-dolomites comprise a series (of variable thickness—up to 530 m) of brecciated and recrystallised siliceous dolomites^{2,3}. Early diagenetic gypsum and anhydrite have been replaced by quartz, dolomite, calcite and pyrrhotite. Rare minute anhydrite relics have been identified in some dolomite pseudomorphs after gypsum. Similarly, pseudomorphed sulphate evaporites have recently been reported from the McArthur Group¹⁰ (1,600–1,500 Myr BP) and are also found in the Paradise Creek Formation¹ (1,600–1,500 Myr BP) (M. Muir, personal communication).

The sequence at Mount Isa has undergone several deformations^{3,11} in low (chlorite grade) greenschist facies metamorphic conditions. The deformations were not penetrative and a slaty cleavage is well developed only in the more argillaceous beds. Bedding and primary textures were recognisable in almost all our samples including the coarse grained massive dolomites³. Our samples of the silica-dolomites (collected mainly from the 3,000N cross section¹²) are fine-grained (5–25 μm) laminated dolomitic siltstones, laminated cherts interbedded with coarse-grained (200 μm –2 cm) carbonate layers (Fig. 1a), and massive coarse-grained carbonates. The carbonate minerals are dolomite, ferroan dolomite, calcite, with minor siderite and ankerite. Two types of pseudomorphed sulphate evaporites have been found in the laminated cherts and interbedded coarse-grained carbonates.

The first type of pseudomorph was most easily recognised in the laminated chert layers (Fig. 1b) where they cut across and distort the bedding (Fig. 1b,c) indicating that the original sulphate minerals crystallised in the host sediment before compaction—during diagenesis. The lath-shaped pseudomorphs were up to 4 cm long, with length/width ratios of 10:1

or greater and they had square cross-sections. This habit is characteristic of diagenetic anhydrite. These pseudomorphs now consisted of a mosaic of quartz grains of a size (50–100 μm) which is consistently larger than that of the enclosing chert (Fig. 1c). Such textures indicated that the pseudomorphs have undergone a void phase similar to the pseudomorphs after early diagenetic anhydrite from the Pine Point Pb/Zn deposit¹³.

The second type of pseudomorphs were large (0.1–3 cm) single crystal dolomite replacements after gypsum and were found in the fine-grained (5–25 μm) dolomitic siltstones and in the coarse-grained carbonates. These pseudomorphed crystals had either angular or diskoidal terminations. The diskoidal types had a similar habit to gypsum which had developed interstitially in recent and ancient sabkha environments^{14,15}. The angular pseudomorphs after gypsum are six sided, with interfacial angles similar to those of gypsum elongate parallel to the *c* axis¹⁰. Many pseudomorphs, however, are commonly fractured and brecciated. In some of the dolomite pseudomorphs after gypsum, minute relics (10–20 μm) of anhydrite were found with the petrological microscope, thus indicating a complex replacement history of gypsum–anhydrite–dolomite. This identification was confirmed by X-ray diffraction of carbonate fractions and by scanning electron microscope microanalysis. The preservation of anhydrite relics, of carbonaceous material^{2,3}, the textures and the reduced nature of the sediments^{2,3} indicated that the diskoidal gypsum was of evaporitic origin rather than formed by the degradation of sulphides¹⁶.

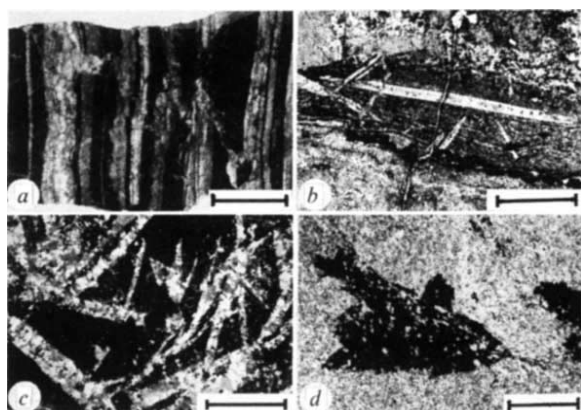


Fig. 1 Photographs of samples from the 3,000 N cross-section, Mt Isa Mine. *a*, Hand specimen of laminated chert (dark layers) and coarse-grained carbonate layers (light) (14 level, bar scale = 2 cm). *b*, Laminated chert with large lath pseudomorphs after anhydrite (plane polarised light, 9 level, bar scale = 5 mm). *c*, Details of pseudomorphs after anhydrite showing quartz mosaic replacements and laths cutting across the bedding (crossed polars with tint plate (12 level, bar scale = 400 μm). *d*, Pyrrhotite grain with diskoidal termination in matrix of fine grained dolomitic siltstone (plane polarised light, 16 level, bar scale = 800 μm).

Other textural features of the silica–dolomite suggested replacement of sulphate evaporites. In many dolomitic siltstones, acicular and diskoidal pyrrhotite grains (Fig. 1d) seem to have completely replaced anhydrite and gypsum. Silicified rosette structures (1–4 mm) and nodules occurred in the cherts. Some coarse-grained carbonate units had fanning and rosette textures, diskoidal terminations, 90° reentrant angles and herring bone textures of included material. Chert layers contain finely laminated carbonaceous material identical to that found in many silicified stromatolites (W. Diver, personal communication) and as such were interpreted as cryptalgal^{2,8}.

Carbonaceous material is also found in abundance associated with framboidal pyrite¹⁷ in the silica–dolomite and in the Urquhart Shales. The nature of the pseudomorphs, the relict anhydrite, the textures and the petrological characteristics of the sediments at Mount Isa¹⁸ are similar to those found in sabkha–intertidal environments^{14,15,19,20}.

In our studies, evaporitic features were found over a thickness of 80 m and down dip for 500 m (from levels 9 to 16 in the mine). Determination of the full extent of the evaporites was hampered by limited sampling and because in some places the original sedimentary and diagenetic textures were obscured by later deformation and metamorphism.

The recognition of a significant volume of sulphate evaporites at Mount Isa is an important addition to the growing number of occurrences of such deposits which are at least 1,500 Myr old (ref. 10) or older (refs 21–23). This widespread occurrence of early and mid Proterozoic sulphate evaporites is a clear contradiction of Cloud's model²⁴ of atmosphere–hydrosphere evolution in which such deposits are held to be insignificant before 1,000 Myr BP.

Furthermore, the Mount Isa evaporites have significant metallogenic implications. Various models for the formation of stratiform lead–zinc and copper ores involving sulphur and metal rich evaporitic brines have been proposed^{4–6,13}. Our interpretation of the deposition of the silica–dolomite in a marginal sabkha environment and the abundance of carbonaceous material in the Mount Isa ores^{2,3} is consistent with a single stage sabkha brine model⁶ involving bacterial reduction of sulphate for the fixation of the metals. This hypothesis contrasts strongly with the view that the Mount Isa deposit is of volcanogenic origin^{2,3,8,25}. Although a volcanogenic model cannot be unequivocally refuted, the recognition of replaced evaporites at both McArthur River¹⁰ and at Mount Isa raises the possibility that these deposits may have been formed from metal rich brines of the kind forming today in evaporitic basins⁷.

We thank Drs W. Diver, M. Muir, D. Shearman and H. Clemmey for stimulating discussion and criticism and Mount Isa Mines for access to material and for support for D.G.C.

K. R. MCCLAY

D. G. CARLILE

Department of Geology,
Imperial College,
London SW7, UK

Received 11 January; accepted 18 April 1978.

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Prehnite–pumpellyite facies metamorphism in County Cavan, Ireland

PREHNITE and pumpellyite have been found in County Cavan, Ireland. They coexist over an area of 120 km² and occur with combinations of quartz, calcite, albite, chlorite, epidote, phengite, sphene and celadonite in pre- and post-cleavage veins, in the matrix of Lower Palaeozoic meta-volcanic greywackes and also as amygdale fillings and phenocryst pseudomorphs in spilites inter-bedded with the greywackes. These assemblages are interpreted as having formed during low-grade metamorphism. This is apparently the first substantiated report of prehnite–pumpellyite facies regional metamorphism in the British Isles to be accompanied by complete analysis of the critical mineral pair. Until recently^{1,2} coexisting prehnite–pumpellyite occurrences in Britain have not been interpreted as metamorphic indicators.

The prehnite–pumpellyite facies has a worldwide distribution³. Metamorphic assemblages containing prehnite and/or pumpellyite have been reported from Lower Palaeozoic rocks throughout the northern part of the Appalachian region⁴ (see Fig. 1). It is anomalous that a similar regional low-grade mineral facies has not been described from the equivalent Paratectonic Caledonides of the British Isles, and this is despite published occurrences of the critical minerals from Scotland^{5,6}, Wales^{7,8} and Ireland⁹ (see Fig. 1).

I am mapping 120 km² of the Longford-Down Massif in County Cavan. The main features of stratigraphy and structure are summarised in Fig. 2 and the petrography of the various formations is given in Table 1. The dominant rock in the psammitic formations is a very proximal thick-bedded lithic greywacke with thin silty or shaly partings. Feldspathic volcanic rock fragments are the most abundant; detrital clinopyroxene, glaucophane and spilite fragments are confined to the Oldtown Psammitic Formation. Recently rock fragments containing glaucophane ± garnet ± white mica ± epidote ± chlorite ± sphene assemblages have been described in greywackes directly along strike in County Longford¹⁰. The pelitic formations in County Cavan are rhythmically bedded feldspathic greywackes and shales showing the typical features of distal turbidites.

A brecciated vesicular lava has been described from near the top of the Loch Acanon Pelitic Formation¹¹. I have mapped this several kilometres north and south of Loch Acanon and recognise it as the Drumcalpin Spilite Member. It seems to be a tuff breccia which has slumped into place.



Fig. 1 Prehnite and/or pumpellyite-bearing metamorphic assemblages in the Appalachia–Caledonian Orogen. 1–7, Prehnite and/or pumpellyite occurrences in Ordovician rocks of the Appalachians⁴. 8, Pumpellyite and actinolite in Loch Nafuoey Spilites, County Mayo⁹. 9, Pumpellyite in altered granophyre from Curlew Mts, County Mayo¹⁷. 10, Prehnite and/or pumpellyite bearing assemblages from County Cavan. 11, Prehnite and pumpellyite in spilite from Lambay Island Porphyry. 12, Pumpellyite in spilite pillow lava from Ballantrae Igneous Complex⁵. 13, Prehnite and pumpellyite in veins and groundmass of Portpatrick Group greywackes⁶. 14, Prehnite and pumpellyite from dolerites in the Upper Conway Valley⁷. 15, Prehnite and pumpellyite from the Builth Volcanics⁸ (spilites). 16, Prehnite and pumpellyite in the Fishguard Volcanic Group, Pembrokeshire². 8–16 Are all Ordovician. Zone A, northern Orthotectonic Zone²⁰ (Zone C of refs 21, 22). Zone B, Paratectonic Zone²⁰ (Zone E of refs 21, 22). Zone C, southern orthotectonic Zone²⁰ (Zones G and H of refs 21, 22). (Pre-Atlantic reconstruction after that of Bullard *et al.*²³)

The petrography and sedimentary current directions indicate a source region lying to the north comprising an active calcalkaline volcanic arc, a blueschist facies terrain and plutonic granitic rocks.

The psammitic units are generally uncleaved although thin sections show how deformation has elongated the clastic fragments and aligned the choritic–sericitic matrix. The pelitic units display cleavage; some of the finer grained shales are slates. The spilites are vaguely cleaved. The junction between the Oldtown and Slieve Glah Formations is the 200 m wide Slieve Glah Shear Zone, which has a distinctive mylonitic fabric.

Apart from the primary detrital minerals in the greywackes and the primary euhedral clinopyroxene in the spilites, the rocks have a distinctive assemblage of secondary minerals including albite, calcite, chlorite, quartz, phengite, prehnite, pumpellyite, sphene, epidote and celadonite. Localities where prehnite and/or pumpellyite have been identified are given in Fig. 2. Secondary actinolite and zeolites have not been found.

I have analysed a selection of rocks with an electron probe and the results of over 500 mineral analyses will be detailed

Table 1 Petrography of County Cavan Formations

Unit	Lithic fragments	Detrital minerals	Metamorphic minerals
Oldtown Psammitic Formation	Hornblende andesite, spilite, rhyolite, felsite tuff, granite, biotite–granodiorite, hornblende–granodiorite, gabbro, quartzite, shale, siltstone	Quartz, feldspar, hornblende, biotite, white mica, clinopyroxene, glaucophane, garnet, spinel	Quartz, calcite, chlorite, albite, prehnite, pumpellyite, phengite, sphene, epidote, celadonite, sulphide, graphite
Slieve Glah Pelitic Formation	Granite, rhyolite, felsite tuff, shale, silt	Quartz, feldspar, biotite, white mica	Quartz, calcite, chlorite, sphene, phengite, epidote, sulphide, graphite
Carrickallen and Drumcora Psammitic Formations	Granite, granodiorite, rhyolite, hornblende andesite, shale, siltstone	Quartz, feldspar, biotite, white mica, hornblende	Quartz, calcite, chlorite, sphene, phengite, epidote, celadonite, sulphide, graphite
Loch Acanon Pelitic Formation	None	Quartz, feldspar	Quartz, albite, calcite, white mica, chlorite, graphite, sulphide
Drumcalpin Spilite Member	Spilite cobbles	Primary igneous clinopyroxene, plagioclase, Fe–Ti–oxide (?)	Albite, chlorite, phengite, quartz, prehnite, pumpellyite, sphene, celadonite

elsewhere. Nearly all the feldspar in greywacke and spilite is albite (An_{5-0}). Only two analyses of calcic plagioclase were found (An_{26} , An_{16}). Refractive index comparisons with quartz in many thin sections show that the plagioclase is very sodic. Albite is commonly full of microfluid inclusions and sericite flakes. An abundance of calcite veins and calcite matrix in greywacke and spilite indicates appreciable Ca transfer and supports a metamorphic origin of albite by breakdown of detrital plagioclase. The calcite shows insignificant Mg, Fe or Mn substitution and density tests on crystals preclude the presence of aragonite.

Chlorites occurring as matrix in greywacke and amygdale fillings and groundmass replacement in spilite are either pycnochlorite or diabantite. Sericite replacing albite is usually phengite with tetrahedral Si:Al ratios greater than 4:1 although occasional muscovites do occur. The detrital white micas in the psammitic formations are also mainly phengite with occasional muscovite. The greywackes are cemented with a very fine inter-growth of chlorite, sericite, quartz and carbonate which is interpreted to be the metamorphosed clay matrix.

Platy prehnite analysed in a shatter vein (GOC 3, Fig. 2) has the formula (based on 22 O) $Ca_{3.97} Fe_{0.03} Ti_{0.03} Al_{3.96} (Al_{0.02} Si_{5.98}) O_{20}(OH)_4$. Coexisting colourless prismatic pumpellyites in the same vein have the formulae (based on Σ cations = 16.0) $Ca_{3.83-3.95} (Fe_{0.41-0.48} Mg_{0.55-0.51} Al_{0.89-1.02}) Al_{4.0} Si_{6.31-6.01} (O, OH)_{28}$. Prehnites, pseudomorphing olivine, have been analysed in a spilite (JK34, Fig. 2) and have the formulae $Ca_{3.66-3.59} Fe_{0.60-0.42} Ti_{0.00-0.02} Mg_{0.17-0.18} Al_{3.65-3.79} Si_{6.04-6.05} O_{20}(OH)_4$. Brown pumpellyite in sericitised albite pseudophenocrysts in the same rock was too fine grained and spongy to be analysed; however, successful analyses were made on a

similar bright green spongy pumpellyite in another spilite (GOC 67, Fig. 2). Formulae are $Ca_{3.86-3.48} (Fe_{2.01-0.96} Mg_{0.12-0.68} Al_{0-0.42}) Al_{3.87-4.00} Si_{6.14-6.32} (O, OH)_{28}$. The Al-rich pumpellyites from Cavan plot in the same field on a $Al^{3+}:(Fe^{2+} + Fe^{3+}):Mg^{2+}$ triangular diagram¹² as Al-rich pumpellyite from Californian glaucophane schists. The Fe-rich pumpellyite from the Cavan spilites compares well with New Zealand Upper Wakatipu Zones I and II and Canadian Vancouver Island pumpellyites of presumably lower pressure genesis¹². Interestingly, the high-pressure Al-rich pumpellyite from Cavan (which occurs in pre- and post-cleavage veins cutting greywackes with detrital glaucophane, phengite and (?) albite) is separated from the lower pressure Fe-rich pumpellyite bearing spilites by the Slieve Glah Shear Zone.

Estimates of depth of burial of the Cavan Upper Ordovician-lowermost Silurian rocks (see Fig. 2) are difficult as the stratigraphy is so poorly exposed and since it is probably cut up into thrust imbricate wedges¹³. Estimates suggest a maximum of 5 km and a minimum of 3 km of Silurian sedimentation in any one area of County Down (T. B. Anderson and T. D. Cameron, personal communication). These depths of burial are quite compatible with other terrains where prehnite-pumpellyite facies metamorphism has developed¹⁴⁻¹⁶. Tectonic burial is not precluded (see Fig. 3 of McKerrow *et al.*¹³).

I have tentatively identified pumpellyite in altered granophyre from the Curlew Mts Anticline¹⁷, County Mayo; pumpellyite in the Carrickatee (spilite) Member¹⁸, and prehnite and pumpellyite in the Lambay Island Porphyry¹⁹ and confirmed the presence of prehnite and pumpellyite in veins and the groundmass of Portpatrick Group greywackes from Scotland⁶. These, together with the published accounts shown on Fig. 1 suggest a regional distribution of prehnite-pumpellyite facies metamorphism in Ordovician rocks from throughout the Paratectonic and southern Orthotectonic Caledonides of the British Isles.

I thank P. Morris, K. O'Hara, P. O'Neill, I. Sanders, M. Stanley and C. Stillman for samples, J. Long and N. Charnley for providing microprobe facilities at Cambridge, Professor D. S. Coombs for useful comments; Professor J. C. Brindley for suggesting the area for study and for providing material (collected by E. Farley, P. Tennet, J. Kavanagh), and J. Booker for typing. This work was funded by Tara Mines Ltd, Ireland.

GRAHAME J. H. OLIVER

Department of Geology, University College,
National University of Ireland, Belfield,
Dublin 4, Ireland

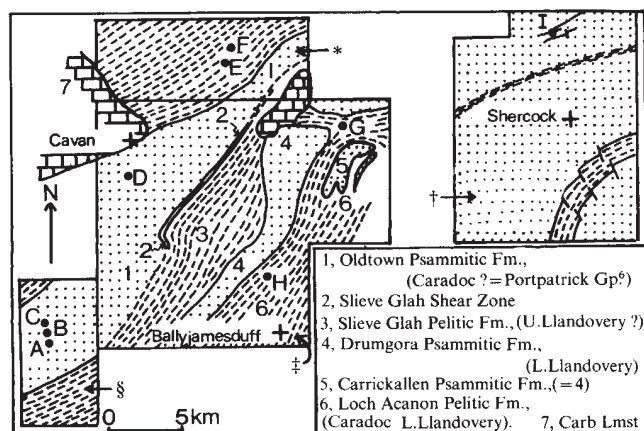


Fig. 2 Geological map of central County Cavan with prehnite and/or pumpellyite locations. A, sample KO1, prehnite + colourless pumpellyite in matrix of greywacke from unit 1 (from National Grid Reference M369903). B, sample KO2, prehnite + colourless pumpellyite vein in greywacke from unit 1 (NGR M368910). C, sample KO3, quartz + colourless pumpellyite in vein in greywacke from unit 1 (NGR M366913). D, sample GOC 3, Fe-poor prehnite + Al-rich pumpellyite + quartz + calcite in shatter vein in unit 1 (NGR H425025). E, sample PO82, prehnite + colourless pumpellyite + quartz in precleavage vein, microfold axes of vein parallel regional cleavage (NGR H480109). F, sample PO71a, colourless pumpellyite vein cross-cutting unit 1 (NGR H485122). G, sample JK34, blue-green pumpellyite + Fe-rich prehnite in spilite cobble (NGR H555361 and many nearby localities). H, sample GOC67, Fe-rich pumpellyite + Fe-rich prehnite in spilite cobble (NGR N498940). I, sample GOC89, light brown pumpellyite in spilite cobble in the Carrickatee Member¹⁸ (NGR N750130). Age of units 1-6 based on graptolites (Phillips and Skevington¹¹ and M. H. Eales, personal communication). *, Mapped by P. O'Neill (unpublished); †, mapped by O'Connor¹⁸; ‡, mapped by G. J. H. Oliver; §, mapped by K. O'Hara (data unpublished).

Received 11 April; accepted 23 May 1978.

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Rates of chemical denudation of silicate rocks in tropical catchments

CHEMICAL denudation includes the set of processes which mobilise ions from rocks and carry them to the sea in solution. These processes, and the rates at which they act, are of considerable importance in the development of landforms and soils because they are responsible for weakening rocks which then become susceptible to soil formation and erosion. However, very few adequate measurements of such rates have been made. I report here a set of such measurements from tropical catchments in Kenya. They are the first set of reported values that correctly measure the rate of chemical denudation in the tropics because they include only true denudation components¹ rather than biogenic and atmospheric inputs. Even for temperate regions I am not aware of a set of such data over a range of climate from wet to dry. The data presented here indicate how chemical denudation varies over such a gradient.

Chemical denudation is usually studied by measuring the solute concentration and the water discharge of streams, and the rate is usually expressed in tonnes $\text{km}^{-2} \text{yr}^{-1}$ of drainage basin. The estimation of chemical denudation rates is complicated by several factors. Both the discharge and the concentration of solutes in streams fluctuate with time; the former over two or more orders of magnitude during a typical year, the latter by two- or threefold. It is necessary, therefore, to have a good record of the two variables over many years. Livingstone² has emphasised that such records are not generally available in the tropics. The few rivers for which data are available on a systematic basis (for example, the Amazon³ and the Mekong⁴) are large, and drain catchments with a variety of rock types, climates and land uses.

Janda¹ has indicated another complication in interpreting chemical denudation rates from streamwater chemistry. The gross solute concentration includes bicarbonate, carbonate, sulphate, and chloride ions, at least part of which must have entered the drainage water either directly from the atmosphere or during the solution of carbon dioxide, for example, in soil pores. These atmospheric and biogenic anions should not be counted as chemical denudation of the rock. Many sedimentary rocks contain these anions and it is not possible to separate the amount that is added to streamwater by chemical denudation. Such a separation is possible with rocks composed entirely of silicate minerals and only silicate rocks are dealt with here.

Precipitation and dry fallout contribute cations to the drainage basin and part of these must be subtracted from the gross solute concentration of streamwater to compute denudation rates. Even if precipitation chemistry were known, however, it is not clear how much of the cation input should be subtracted from gross solute concentration in continental regions because most of the cations in rainfall and dry fallout there probably originate from dust and burning vegetation and therefore represent a true component of chemical denudation when the cations are leached from the catchment¹.

A few recent investigations have incorporated the necessary corrections⁵⁻¹², but apart from Moberly's¹³ data for eastern Oahu, tropical data are not amended. The corrected values of chemical denudation rate range from 1.1 to 72.2 tonnes $\text{km}^{-2} \text{yr}^{-1}$, but because the few measurements sample such a wide range of lithology and climate, it is difficult to generalise about the variation of chemical denudation rate in the same way that Langbein and Dawdy¹⁴ and Meybeck¹⁵ did for gross solute yields.

During two average wet seasons and two dry seasons of a single hydrological year, water samples were collected at monthly or biweekly intervals at 70 river gauging stations throughout the part of Kenya that is drained by perennial

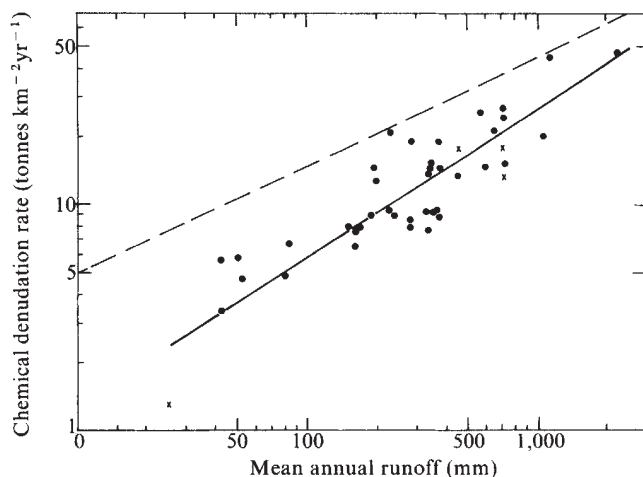


Fig. 1 Relationship between the rate of chemical denudation and mean annual runoff for 43 Kenyan river basins. ●, catchments with a good daily flow record; ×, basins for which the runoff was estimated from other hydrological data. The solid regression line is calculated for only 30 of the basins which are independent of one another; that is, tributary and downstream stations in the same basin were not used together. Chemical denudation rate = $0.28R^{0.66}$; $n = 30(43)$; $S_{y,x} = 0.13$ log units. Dashed line indicates gross solute yield and is based on measurements at 44 independent stations among the 70 sampled.

streams¹⁶. Most of the streams drain single rock types which are all igneous and metamorphic silicates, containing only an occasional trace of carbonate, sulphate, or chloride. The lithologies sampled include gneiss, basalt, andesite, trachyte, rhyolite and phonolite, each being represented by at least two basins with a single rock type. Most of the more than 100 chemical analyses of these rocks made by the Kenya Mines and Geology Department showed zero concentrations of carbonate, sulphate and chloride. Mean annual air temperatures varied with elevation from 10°C to 30°C. Annual rainfall also varied with elevation, and ranged from 250 mm to 3,000 mm; the vegetation cover varied accordingly from grass and bush, through cultivated crops to dense evergreen forest, and had been generally stable during the previous decade. The use of chemical fertilisers containing petrological components was limited and runoff processes¹⁷ in most of the fertilised areas were unlikely to carry large quantities of petrological components into streams in solution.

The specific conductance (μS at 25°C) of each of the 850 samples was measured and used with a local empirical relationship between specific conductance and total dissolved solids, to estimate the concentration of dissolved solids. Daily flow records extending over 10–15 yr were available for almost all of the sampling stations, and were used with average concentrations over the one-year sampling period to calculate gross solute yields. These yields increased with runoff, as shown in Fig. 1, and were lower than the average solute yields reported by Langbein and Dawdy¹⁴ for temperate regions. Their data were influenced by solutes from sedimentary rocks and by large inputs of pollutants from agriculture and industry¹⁸.

Analyses of individual solutes were carried out on 175 of the water samples. The constituents measured were dissolved silica (expressed as SiO_2), Na^+ , K^+ , Mg^{2+} and Ca^{2+} . Silica originates almost solely from the rocks¹⁹, but some of the four cations came from rainfall and from dry fallout. The portion of the atmospheric input that was derived from the ocean had to be subtracted, because solutes derived from terrestrial dust and burning vegetation should be considered as true components of chemical denudation, even when they enter the catchment in solid form from other regions¹.

It was assumed that the atmospheric salt concentrations derived from the ocean retain the same relationship to one another as existed in seawater. This is a reasonable first approximation for the tropics where data on precipitation chemistry are scarce. The only cation value significantly affected by this assumption is the concentration of Na, and Eriksson¹⁸ has shown that chloride is washed from the atmosphere mainly as NaCl, a fact which strengthens the validity of the temporary assumption used here.

The oceanic contribution to streamwater concentrations can then be computed assuming that rain-supplied chloride ions are leached quickly out of a catchment (this has been adequately demonstrated elsewhere¹⁹). The calculation is based on the equation

$$\frac{[\text{Cl}^-]_{\text{stream}}[\text{Cation}]_{\text{sea}}}{[\text{Cl}^-]_{\text{sea}}} = [\text{Cation}]_{\text{evap. rain}}$$

where the brackets refer to average concentrations and evap. rain indicates the cation concentration in rainwater after it has been concentrated by evaporation.

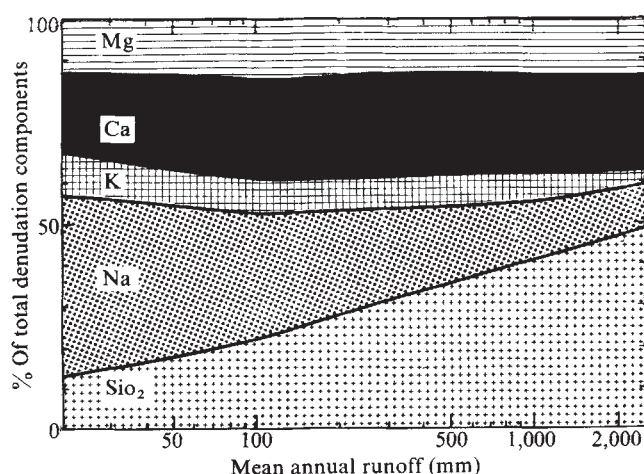


Fig. 2 Proportion of the total denudation contributed by each component in different climates. At each abscissa value the full centig scale represents 100% of the denudation components dissolved from a catchment. The shaded fields indicate the proportion contributed by the various solutes. The proportions, expressed in terms of equivalents, were calculated from the equation in Table 1.

The average atmospheric inputs were subtracted from the average concentration of each cation in the streamwater to obtain the average concentration released by rock weathering. This last value for each component correlated significantly with mean annual runoff from each basin (see Table 1). The yields of silica, sodium, potassium, calcium and magnesium were then calculated from their average concentration and mean annual runoff. The sums of these yields, shown in Fig. 1, indicate that the rate of chemical denudation increased approximately as the two-thirds power of runoff. As Janda¹ predicted, using gross solute transport to estimate denudation rate led to an exaggeration of ~1.5–2.5 times the true value.

Runoff is the dominant variable affecting solution rates. It is associated with the general level of biological activity in the soil and therefore the partial pressure of carbon dioxide in solutions that percolate through rocks and soils, as well as the volume of water available for dissolution. Runoff, in turn, is

affected by precipitation, net radiation, air temperature and vegetation cover. Differences in annual solution rate between rock types for any value of runoff in Fig. 1 are minor and sometimes contradictory. The wide range of lithology (from basalt to rhyolite, for example) does not have a major effect on the denudation rate.

In addition to controlling the absolute amount of dissolution in a catchment, the amount of available moisture affects chemical weathering processes in ways which release varying amounts of solutes in different climates. The effect of these processes is shown in Fig. 2, in which the proportion that each solute contributed to the total denudation is expressed by a shaded fraction of the graph at each runoff value. The proportions were calculated from the equations in Table 1. In dry climates, Na is the most common solute and the SiO_2 :Na ratio (in terms of equivalents) is 0.30 where the mean annual runoff is 20 mm. As the climate became wetter, the SiO_2 :Na ratio increased to 4.33 at 2,000 mm. The proportions of other cations remained relatively stable through the range of climate, and the ratio of silica to total cations increased from 0.15 to 0.91 over the same range of runoff.

These field variations can be explained by interpreting laboratory investigations of clay mineral stability^{20–22} in the light of hydrological conditions in Kenya. In the wet highland regions, weathering of primary silicates, particularly Na-feldspar and ferromagnesian minerals from phonolite, the most common rock, releases cations and dissolved silica (H_4SiO_4). Leaching is relatively rapid, and the residence time of water in the soil is too short to allow high concentrations of silica or metal cations to build up in the matric solution²³. In these conditions, kaolinite is the most common stable product of weathering, and the streamwaters of wet regions in Kenya plot within the kaolinite field on stability diagrams^{20,21,23} based on activities of H_4SiO_4 , various cations, and pH (5.5–7 in groundwaters of these regions). In these conditions most of the silica and almost all of the Na^+ (for example) are leached from the profile.

In drier climates, the residence time of water in the regolith is greater, allowing time for the development of higher solute concentrations in the matric solution. Further concentration takes place by evaporation during the dry season. These two processes also lead to high pH values (7–8.5), and the drainage waters of regions with 20 mm of runoff plot in the stability field of Mg-montmorillonite²², and close to the kaolinite-montmorillonite boundary for sodium and calcium clays^{20,21}. Montmorillonites become common on the poorly drained footslopes in regions of Kenya with 20 mm of runoff per year, and occur even more extensively in the drier regions where extrapolation of the equations in Table 1 predicts the occurrence of montmorillonites. For Na-montmorillonite to form (for example) SiO_2 and Na^+ must be immobilised in the ratio of 11:1 (ref. 21), allowing a relatively small amount of dissolved silica to escape in the streamwater.

Table 1 Regression equations relating mean annual runoff to average concentrations (mg l^{-1}) of solutes from rock weathering in Kenya

Solute	Equation*
Silica	$[\text{SiO}_2] = 51.9R^{-0.16}$
Sodium	$[\text{Na}^+] = 380R^{-0.74}$
Potassium	$[\text{K}^+] = 77.8R^{-0.54}$
Calcium	$[\text{Ca}^{2+}] = 57.2R^{-0.41}$
Magnesium	$[\text{Mg}^{2+}] = 23.2R^{-0.43}$
Total denudation components	$[\text{TDC}] = 258R^{-0.33}$

The sample size for each regression was 30 independent catchments. All of the correlations are significant at the 0.01 level.

* R is the annual runoff in mm.

Fieldwork for this study was supported by the McGill-Rockefeller Program. Records of stream discharge were provided by the Kenya Ministry of Water Development. Analyses of individual solutes were carried out at the laboratories of the Ministry of Agriculture, the Ministry of Works and the Government Chemist, Nairobi.

THOMAS DUNNE

Department of Geological Sciences
and Quaternary Research Center,
University of Washington,
Seattle, Washington 98195

Received 16 January; accepted 9 May 1978.

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Zooplankton fecal pellets and element residence times in the ocean

THE concept of oceanic residence time has been used widely in marine chemistry; element residence times¹ range from 10⁸ to <100 yr, and provide a useful measure of the reactivity of an element in the ocean. The residence time of an element is defined as the average time an element spends in ocean water between introduction into the ocean and incorporation into the sediments. Between introduction and incorporation transport mechanisms must exist; a relationship between residence time and a property associated with a particular transport mechanism is, therefore, a logical possibility. A transport mechanism which has received increasing attention and which is discussed here involves zooplankton fecal pellets. These pellets sink fairly rapidly^{2–4}, at rates ranging from 50 to ~950 m per day, and they decompose relatively slowly. They seem to have the potential to reach the sea bottom in most areas, and have been identified in sedimentation traps³ placed at depth in the ocean; moreover, a model for particle settling in the equatorial Atlantic Ocean⁴ indicated that 99% of the vertical mass flux through 388 m is carried by fecal matter and fecal pellets. As far as specific elements are concerned, fecal pellets have been shown to be important in the vertical oceanic transport of zinc⁵, cerium⁶, polonium⁷ and plutonium⁸.

Determinations of element concentrations in the fecal pellets from a common zooplanktonic species, the euphausiid *Meganycitiphanes norvegica*, are now available for 18 elements⁹. When we plotted the log of the concentration factor of each element in fecal pellets relative to seawater against the logarithm of the oceanic residence time of the element a clear relationship was obtained. The relationship was linear, of the form:

$$\log F = \alpha + \beta \log T \quad (1)$$

where $F = C_f/C_{sw}$, C_f being the element concentration in wet fecal pellets in $\mu\text{g per kg}$ and C_{sw} the concentration in seawater in $\mu\text{g l}^{-1}$ and T the oceanic residence time in years. C_f values were taken from published data⁹ plus our unpublished value for uranium; a wet to dry ratio of 4.4 was used for fecal pellet material^{8,9}; C_{sw} values were taken from Goldberg *et al.*¹

The residence time, T , is calculated from the formula:

$$T = A/(dA/dt) \quad (2)$$

where A is the total amount of the element in the oceans and dA/dt is the amount introduced into the oceans per unit time. The residence time concept assumes a steady-state system in which the amount of an element entering the marine environment is compensated by the transfer of an equivalent amount from seawater to the sediment; dA/dt is, therefore, the amount precipitating per unit time as well as the amount introduced per unit time. Residence times can accordingly be calculated in two ways. Goldberg *et al.*¹ used data for dissolved element concentration in river waters to calculate the input dA/dt and hence T ; alternatively, one can use element concentrations in marine sediments to calculate T from output dA/dt . It is important to check whether or not the fit to equation (1) is sensitively dependent on the method used for calculating T ; accordingly, we calculated the fit twice: the first time using the Goldberg *et al.*¹ ('river input') $\log T$ values, and then using 'sediment output' $\log T$ values calculated by rewriting equation (2):

$$T = \frac{C_{sw}}{C_{sed}} (3.8 \times 10^5) \text{ yr} \quad (3)$$

In equation (3) C_{sed} is the element concentration in sediment in $\mu\text{g per g}$ and the numerical factor 3.8×10^5 comes from assuming a total mass of seawater in the oceans of 1.4×10^{21} kg, an average density of seawater of 1.028 kg l^{-1} and a mass output to the sediment of $3.6 \times 10^{15} \text{ g yr}^{-1}$. Values for C_{sed} were taken from Chester and Aston¹⁰ for deep-sea clays, and values for C_{sw} are from Goldberg *et al.*¹ Both calculations were performed for the same 15 elements which represented all the elements for which the necessary data were available, in the references cited, for all four of fecal pellets, seawater, river water and sediments. In equation (1) there is no *a priori* reason why one of the two variables $\log F$ and $\log T$ should be designated as the independent variable; moreover, neither are known without error. Classical regression cannot therefore be used to quantify the linear relationship¹¹, and we have instead calculated the reduced major axis line¹². The parameters α and β thus obtained are given in Table 1. If $\log T$ is calculated from the sediment output data the correlation is less satisfactory than if it is calculated from the river input data. Furthermore, the two lines are significantly different at the 5% significance level. Figure 1 shows the reduced major axis fit to the river input data points.

At this stage we should draw attention to an inherent inconsistency between the two methods we have used for calculating T . The residence time concept is usually assumed to apply to dissolved elements, and dissolved element concentrations in river waters were used by Goldberg *et al.* to calculate the river input T . On the other hand, the total element concentration in the sediment represents dissolved and particulate contributions and it is not possible to distinguish between the two fractions. Bewers and Yeats¹³ have taken account of nearshore element precipitation and of the particulate as well as the dissolved river discharges of elements, and were able to obtain improved agreement between river input and sediment output T for seven metals. Six of these seven were elements which were included amongst the 15 used in Table 1; recalculating these fits after substituting the Bewers–Yeats data for T and C_{sw} for these six elements, adding in the seventh Bewers–Yeats element (Cd) as well, and retaining the original data for the other nine elements results in the α , β and

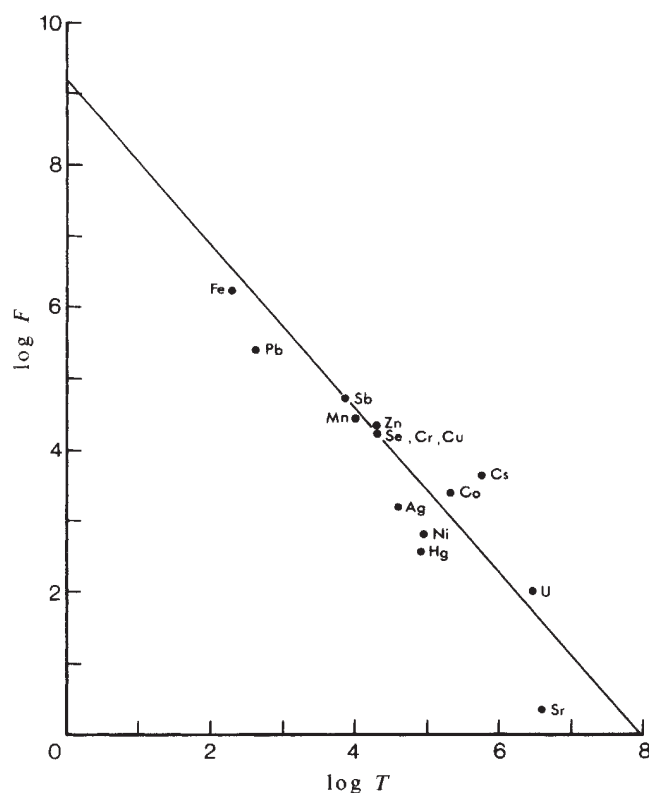


Fig. 1 $\log F$ plotted against $\log T$, using T values calculated from the river input data. The straight line is the reduced major axis line fitted to the data as in Table 1.

r values given in Table 2. Such mixing of data from different sources means that Table 2 results must be regarded with reservation. We included them to draw attention to two facts. First, the river input and sediment output lines are no longer significantly different at the 5% level, indicating that the inherent inconsistency referred to above might be capable of resolution once Bewers-Yeats type calculations have been performed for all the elements involved. Second, a fairly major change in the experimental value for C_{sw} for an element does not necessarily upset the fit to equation (1) because change in C_{sw} results in a change in F as well as in T . The effect on Fig. 1 of changing over to the Bewers-Yeats data for six elements results in little more than these six points moving diagonally upwards along the line.

Although a relationship clearly exists between $\log F$ and $\log T$ it does not necessarily follow that this is one of cause and effect; it is possible that it reflects no more than the mutual dependence of both F and T on the chemical reactivity of the element. Other variables besides F which depend on chemical reactivity might then show an equally satisfactory correlation with T if fitted to the same equation, and we used data⁹ for element concentrations in whole zooplankton (euphausiids) and their moults to investigate this possibility. Fits of $\log Z$ against $\log T$ and $\log M$ against $\log T$ were calculated, where $Z = C_z/C_{sw}$, and $M = C_m/C_{sw}$, C_z and C_m being the element concentrations in whole euphausiids and in moults respectively. Significant correlations were observed, but were less impressive than those listed in Tables 1 and 2; values of r ranged from -0.57 to -0.80 and from -0.63 to -0.91 for the fits of $\log Z$ and $\log M$ respectively. This does not prove the significance of the $\log F$ against $\log T$ relationship, but it convinced us that the relationship involving fecal pellets was more promising than the other two and that it warranted further investigation.

We tried next to develop a theoretical model which would predict our observed equation (1), and started off with an

extreme assumption—that zooplankton fecal pellets provide the only route for the vertical oceanic transport of the elements to the sediments. We start by using equation (2), and considering dA/dt as the rate of loss to the sediment, hence:

$$dA/dt = C_t M_t B \quad (5)$$

where M_t is the wet mass of fecal pellets transported per year to unit area of the ocean bottom, and B is the total area of the ocean bottom. The numerator A in equation (2) is given by:

$$A = C_{sw} V_T \quad (6)$$

where V_T is the total volume of the ocean. We then have:

$$T = \frac{C_{sw} V_T}{C_t M_t B} \quad (7)$$

where C_{sw} is in $\mu\text{g l}^{-1}$, V_T in l , C_t in $\mu\text{g per kg wet fecal pellets}$, M_t in $\text{kg wet fecal pellets m}^{-2} \text{ yr}^{-1}$ and B in m^2 , giving T in yr . Assuming an average oceanic depth of $4,000 \text{ m}$, V_T/B is $4 \times 10^6 \text{ l m}^{-2}$; moreover, C_t/C_{sw} is equal to the fecal pellet concentration factor F , and hence:

$$T = \frac{4 \times 10^6}{F M_t} \text{ yr} \quad (8)$$

and therefore

$$\log F = \log \frac{4 \times 10^6}{M_t} - \log T \quad (9)$$

Table 1 Parameters α and β obtained by calculating the reduced major axis line^{11,12} fit to the equation $\log F = \alpha + \beta \log T$

$\log T$: data source	α	β	r	n
River input	9.2 ± 1.1	-1.20 ± 0.25	-0.91	15
Sediment output	7.1 ± 1.4	-0.80 ± 0.22	-0.84	15

F is the element concentration factor from seawater to euphausiid fecal pellets and T is the element oceanic residence time. Also given are the 95% confidence limits on α and β , the correlation coefficients r and the number of elements n used in each fit. The sources of the data for F and T are given in the text.

Table 2 Recalculation of parameters α and β using Bewers and Yeats¹³ values of T and C_{sw}

$\log T$: data source	α	β	r	n
River input	8.3 ± 0.9	-1.03 ± 0.22	-0.90	16
Sediment output	7.8 ± 0.9	-0.85 ± 0.20	-0.87	16

Seven of the 16 elements were involved; for the other nine elements the input data applicable to Table 1 were used.

We have thus obtained equation (1) and we have predicted that the slope $\beta = -1$. We predict $\alpha = \log(4 \times 10^6)/M_t$, and to evaluate this numerically we need to know M_t , the annual transportation rate of wet fecal pellets to the ocean bottom. Wiebe *et al.*³ investigated particulate matter sinking to the deep sea floor at $2,000 \text{ m}$ in the Tongue of the Ocean, Bahamas, using a sedimentation trap with filters to retain falling particles.

They found that "fecal pellets were one readily identifiable component of the material contributing to the organic fraction on the filters"; counts of pellets on filter aliquots suggested an average of 658 pellets $\text{m}^{-2} \text{d}^{-1}$ reaching the trap, but it was pointed out that these counts "may be as much as one order of magnitude too small not only because of the material lost in trap recovery, but also because some of the fecal pellets, being extremely soft, lost their form and could no longer be positively identified as pellets". Taking a fecal pellet volume of $2.2 \times 10^6 \mu\text{m}^3$ and a density of 1.22 g cm^{-3} (ref. 3), we estimate the average mass of a fecal pellet as $2.7 \mu\text{g}$ wet. Multiplying this by 658 pellets $\text{m}^{-2} \text{d}^{-1}$ gives M_f at $0.66 \times 10^{-3} \text{ kg}$ wet fecal pellets $\text{m}^{-2} \text{yr}^{-1}$. The Bahamas area is an 'average' area insofar as zooplankton biomass is concerned, so this value of M_f should not be atypical; we must not forget, however, that it may be as much as one order of magnitude too small in view of the fact that the 658 pellets $\text{m}^{-2} \text{d}^{-1}$ average which were counted may be an underestimate. We can now predict that $\alpha = 9.8$; should the M_f value indeed be an order of magnitude too low, then we predict $\alpha = 8.8$.

The agreement between these theoretical predictions and the values of α and β given in Tables 1 and 2 is remarkably good, although log-log plots are notoriously insensitive and a difference of unity between predicted and fitted values of α implies a discrepancy of an order of magnitude. One would also expect the theoretical prediction for α to be higher than the value obtained from fits to the experimental data because the basic assumption of our model, that zooplankton fecal pellets provide the only route for vertical oceanic transport, cannot be true for all elements. It could, however, be approximately true for some of the elements, and a primary task is apparently to identify those elements for which this extreme assumption is a good approximation. All other elements should lie below the predicted line on the $\log F$ against $\log T$ plot because the addition of alternative transport routes must result in a lower T ; the distance below the theoretical line should in fact be a measure of the relative importance of alternative transport routes *vis à vis* fecal pellets. If we could eliminate the 'other' elements and then refit the experimental data using only those which remain, we would obtain a new α which would, provided the slope remained unchanged, be higher than the α obtained using all the elements for which data are available. It is interesting that using $\log T$ calculated from sediment data gives a worse correlation, as has already been mentioned, and also gives rise to lower values of α . These low values imply an unrealistically high fecal pellet deposition rate and possibly reflect the fact that the sediment composition includes a particulate contribution from a source or sources other than fecal pellet material.

A final caveat is needed. The data for C_f , the element concentrations in fecal pellets, were obtained from pellets retrieved within 15 h of production⁹. We used these data in fitting equation (1), whereas the theory, equation (5), requires C_f in the fecal pellets depositing on the ocean bottom. Loss of an element to solution as the pellets sink through the water column is a possibility¹⁴, and such a process could have a significant effect on the profiles of some dissolved elements in the water column. Values of C_f obtained from fecal material collected in traps at various depths in the ocean are needed, and will have to be compared critically and in detail with the values from the material we have used here. Our equation (1) will then have to be refitted using the reassessed C_f data. Despite this reservation, we have obtained a rather good quantitative prediction from a model based on an apparently extreme assumption. This suggests that the oceanic residence times of many of the elements shown in Fig. 1 are indeed controlled primarily by the rate of element transport by fecal pellet deposition.

The International Laboratory of Marine Radioactivity operates under a tripartite agreement between the International Atomic Energy Agency, the Government of the Principality of Monaco and the Oceanographic Institute at Monaco.

This work was also supported by the Marine Pollution Programme of the South African National Scientific Programmes Unit.

R. D. CHERRY

J. J. W. HIGGO

Physics Department,
University of Cape Town,
Rondebosch 7700, Cape,
South Africa

S. W. FOWLER

International Laboratory of Marine Radioactivity,
Musée Océanographique,
Principality of Monaco

Received 29 March; accepted 17 May 1978.

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Fauna of fossil mammals from the Miocene of Saudi Arabia

DESPITE several reports of fossil vertebrates from the Arabian region¹, Tleel's² brief reference to fossil mammals in the Dam Formation^{1,3,4} near Dhahran was the first published reference to Tertiary mammals, even though specimens of *Platybelodon* have been included in oil company collections since the 1930s. In 1974 we found two small faunas in the region around Ad Dabtiyah and at Jabal Midra ash-Shamali (Fig. 1). This material was added to specimens already recovered by oil company geologists and we describe these findings here. These faunas are very important as they are the first Tertiary mammalian faunas from the Arabian region and they offer the possibility of establishing faunal affinities between the important African and Asian Miocene faunas.

Fossil mammals from a small hill south-east of Jabal Midra ash-Shamali (26°20'51"N, 50°04'45"E) have been briefly described by Tleel². The fossiliferous beds were identified as Basal Dam Formation and consist of a 1-m thick zone of "multicoloured rubble...containing boulders of older

Fig. 1 Map of eastern Saudi Arabia with Dhahran and sites marked.

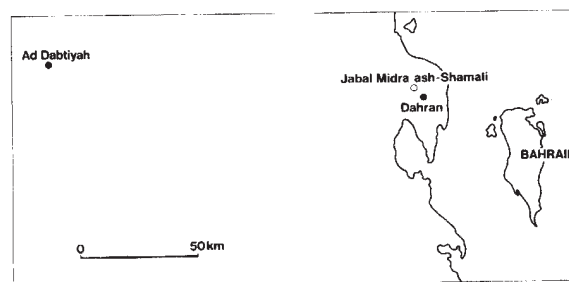


Table 1 The Jabal Midra ash-Shamali fauna

Pisces	<i>Lates</i> sp.	Teeth and bones
Chelonia	Scute fragments	
Mammalia	Lagomorpha	Cheek teeth
	Rodentia	
	cf. <i>Cricetodon atlas</i>	Cheek teeth
	indet.	Cheek teeth
	Artiodactyla	
	Bovidae medium-sized species	Cheek teeth and bone fragments
	small species	Teeth and bone fragments

rocks²². Our samples from this site contain bone and rarer enamel fragments. The rock breaks down slowly in acetic acid solution yielding cheek teeth of rodents and lagomorphs and tooth fragments of two species of bovids in a small and medium size range (Table 1).

The Ad Dabtiyah locality (26°27'02"N, 48°35'24"E) is approximately 4 km from the Sabkha (salt flat) Ad Dabtiyah which is in turn 160 km east-north-east of Dhahran. This locality also lies in the Dam Formation and is probably contemporaneous with the Jabal Midra ash-Shamali locality. Fossil mammals had been discovered at Ad Dabtiyah by geologists of the Arabian American Oil Company who recovered specimens of rhinoceroses and *Platybelodon*, but small and medium-sized mammals were not known. The deposits consist of an ill-graded calcareous pebbly grit containing small pebbles of marly sandy limestone and quartz sand grains in a creamy limy matrix. The palaeoenvironment at this locality was a fluvial coastal plain, perhaps similar to that suggested by Selley⁵ for the Miocene Sirte embayment of Libya. Large algal clumps seem to follow channels in the fossil-bearing deposits. Specimens were recovered from a general surface scatter and from two quarries little more than 250 m apart. Surface and quarried specimens belong to the same species, and no fine distinction is made in the faunal list (Table 2).

The Dam Formation has been dated as middle Miocene, being correlated with the lower Fars Formation of Iraq¹. Kier⁶ suggested a Burdigalian-late early Miocene-age on the basis of echinoids. This may agree with suggestions for the earlier dating of the lower part of the lower Fars⁷. Tleel² suggested a late early-Miocene dating for the formation which would give an age of 14–15 Myr (ref. 8). The fossil mammals fauna may therefore agree in age with the north African Lower Miocene faunas from Moghara⁹, Siwa¹⁰ and Gebel Zelten¹¹, being slightly older than the Beni Mellal¹² and Tunisian¹³ faunas.

Table 2 The Ad Dabtiyah fauna

Pisces	Teeth and bones	Perissodactyla
Chelonia	Scutes	Rhinocerotidae
Crocodylia	Teeth	<i>Brachypotherium</i> sp.
Mammalia		Metapodials and teeth
Primates		<i>Aceratherium/Dicerorhinus</i> sp.
		Jaws, cheek teeth, ankle bones
Hominoidea indet.	2 species	
Teeth and maxilla fragment		Artiodactyla
Carnivora indet.	Phalange	Suidae indet.
		2 species
		Cheek teeth
Rodentia indet.	Incisors	Tragulidae
Hyracoidea		<i>Dorcatherium</i> cf. <i>libiensis</i>
cf. <i>Pachyhyrax championi</i>		Jaws and cheek teeth
Upper and lower cheek teeth		Giraffidae indet.
		Cannon-bone
Proboscidea		Bovidae
<i>Platybelodon</i> sp.		Boselaphinae indet.
Cheek teeth and tusks		Lower cheek teeth and horn-core

East African early Lower Miocene faunas may also agree in age with the Dam Formation mammals, but that of Fort Ternan is probably slightly younger. In the India-Pakistan region the principal fauna that may be relevant is that from Chinji^{8,14,15}.

Although not yet studied in detail, most of the specimens reported here seem to be new at the specific or even generic level and identifications so far suggest agreement with the African rather than the Chinji fauna. This is not surprising as free migration was apparently possible between Arabia and both north and east Africa in Middle Tertiary times¹⁶.

There is agreement at the specific level between the Arabian fauna and some elements in the Beni Mellal, Gebel Zelten and Rusinga faunas. The bovids are not named but agree most closely with those from Beni Mellal and are far more advanced than those from the earlier Gebel Zelten and Rusinga sites. The two hominoids are interesting as they offer the chance of establishing relationships with African and Asian forms. They are described in detail in the following paper¹⁷. With the exception of *Prohylobates*¹⁸ from Moghara, primates are not known from the north African Lower Miocene, but this may reflect conditions of preservation and collecting rather than absence from the faunas. Overall, the Arabian fauna suggests a dating of 15–17 Myr which is rather younger than the earliest east and north African sites but slightly older than Fort Ternan and Beni Mellal.

We thank Drs P. J. Andrews and A. W. Gentry for help with identifications, and Kim Dennis for the figure.

W. R. HAMILTON
P. J. WHYBROW

British Museum (Natural History),
Cromwell Road, London SW7, UK

H. A. MCCLURE

Arabian American Oil Co.,
Dhahran, Saudi Arabia

Received 21 February; accepted 10 May 1978.

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Dryopithecines from the Miocene of Saudi Arabia

WE describe here five dryopithecine specimens from the Dam Formation near Ad Dabtiyah, Saudi Arabia. They were recovered from a calcareous pebbly grit¹, and consist of a maxilla and four isolated teeth. One of the latter is smaller and differs in morphology from the other specimens, which suggests that it may be specifically distinct. The five specimens, which are housed in the British Museum (Natural History) with the registered numbers M35145–35149, have closest affinities with the early Miocene dryopithecines of East Africa^{3,4}.

The most complete specimen, M35145, is a crushed left maxilla with crowns of P³–M² (Fig. 1). The lingual alveolar margins of the I¹–C are also present, but the lateral or buccal alveolus has weathered away. Much of the left side of the palate is present, part of it to the median palatine suture, but it

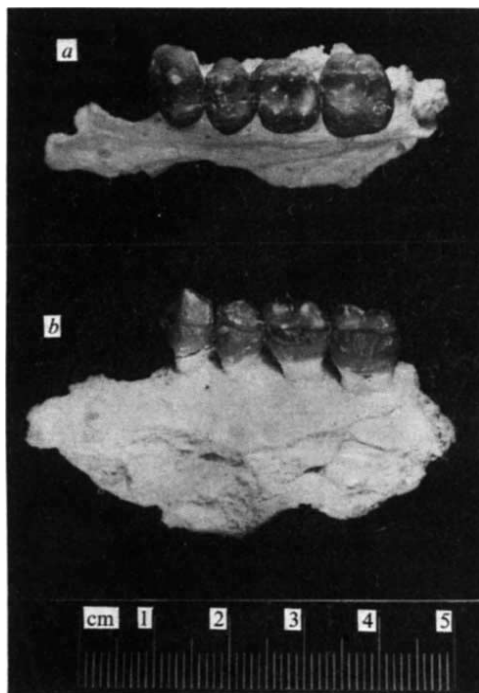


Fig. 1 M35145 occlusal (a) and lingual (b) views.

has been displaced upwards into the floor of the left nasal passage. Before crushing, the palate was probably relatively shallow and narrow, with almost parallel tooth rows. The estimated width of the palate at M^1 is 29 mm, which is slightly narrower than in *Proconsul nyanzae* but much wider than the estimated 20 mm of M16649, the type specimen of '*Sivapithecus africanus*'².

The root sockets of the I^2 and C in M35145 suggest that these teeth were large, but no realistic estimate of size can be made. There is a diastema of 3.1 mm between the canine and lateral incisors. The P^3 is very large, with a strong buccal elongation of the crown. Although the buccal cusp has been chipped at the tip, it is more than twice the height of the lingual cusp, a much greater differential than is present in *Proconsul* specimens or M16649. The P^3 falls within the size range for *P. major*. The P^4 is smaller than the P^3 but both premolars are large relative to M^1 . The premolar-molar proportions for M35145 are beyond the ranges of variation (with one exception) of any east African *Proconsul* species (Table 1).

The molars, like the premolars, are relatively low crowned and have low but massive cusps. They have a prominent lingual cingulum and a smaller buccal one. The lingual cingulum on the M^1 is a shelf-like ridge, and on the M^2 it is smaller and is replaced by a greater degree of lingual flare, as on M16649. The molars are slightly worn, with dentine exposed at the tips of the lingual cusps of M^1 . Enamel thickness on M^2 , the least worn tooth, ranges between 0.6 and 0.9 mm, and on P^4 the thickness is 0.6 mm. These values correspond to those of *Proconsul* species, which range from 0.5 to 1.5 mm. In breadth and length the molars of M35145 fall at the bottom of the *Proconsul nyanzae* size range, again illustrating the difference in crown proportions between this specimen and the three *Proconsul* species: P^3 is in the middle of the range of variation of the largest species, *P. major*, and beyond the intermediate *P. nyanzae* range; M^2 is at the top of the range of variation of the smallest species, *P. africanus*, and smaller than any *P. nyanzae* specimen. A similar proportion exists for M16649 except that overall it is slightly larger than M35145.

One of the four isolated teeth from Saudi Arabia is an M^3 (M35146) from a larger individual than the maxilla; length and breadth dimensions are 10.4 mm and 12.9 mm. The crown is worn down to the level of the massive lingual cingulum, which has been incorporated into the occlusal surface. Two of the other specimens are deciduous teeth which agree in size with the permanent teeth of the maxilla. M35147 is a right dp^4 similar in size and morphology to *Proconsul nyanzae* specimens and M35148 is a dc^1 with a massive lingual cingulum producing a notched appearance for the crown. The lengths and breadths of the teeth are 6.9 mm and 8.3 mm, and 5.9 mm and 4.9 mm, respectively. The remaining specimen, M35149, is a right P^4 . This has a transversely broadened crown, with length and breadth of 5.4 mm and 9.7 mm giving a breadth/length index of 183%. This is considerably smaller and much more mesiodistally compressed than the P^4 of the maxilla which suggests that M35149 may represent a second species of dryopithecine. This must remain only a suggestion until more material is found.

The great importance of these Saudi Arabian specimens lies in their age and their geographical position. The east African early Miocene deposits, where *Proconsul* species are so common⁴, are earlier than the Ad Dabtiyah deposits in Saudi Arabia, and the closest equivalent in east Africa is probably Maboko Island, which is also the only locality from which '*Sivapithecus africanus*' is definitely known^{2,5}. The Saudi Arabian specimens share with '*Sivapithecus africanus*' what is probably a derived character complex in which the premolars are enlarged relative to the molars, but there are also several differences between them, particularly, for instance, the narrow

Table 1 Measurements of dimensions of M35145 compared with M16649, the type specimen of '*Sivapithecus africanus*', and the means of three species of *Proconsul*⁴

	M35145	M16649	<i>P. africanus</i>	<i>P. nyanzae</i>	<i>P. major</i>
P^3 md	7.7	8.5	6.2	7.5	8.9
bl	11.6	12.5	8.9	11.1	12.0
bl/md $\times 100$	150.7	164.2	163.0	163.0	151.6
$P^3/M^1 \times 100$ (module)	96.9	91.8	88.2	88.5	89.5
$P^3/M^1 \times 100$ (bl)	110.3	107.8	96.8	98.3	94.5
P^4 md	7.0	7.0	5.4	6.8	7.2
bl	11.4	12.0	9.2	11.0	12.1
bl/md $\times 100$	163.0	155.8	170.0	163.0	170.0
$P^4/M^1 \times 100$ (module)	94.9	91.6	82.0	89.0	88.0
$P^4/M^1 \times 100$ (bl)	108.4	103.3	100.0	97.1	95.4
M^1 md	8.8	10.2	7.8	9.9	10.9
bl	10.5	11.6	9.2	11.3	12.7
bl/md $\times 100$	119.2	113.8	118.0	115.0	116.0
M^2 md	9.5	(11)	8.5	11.3	12.7
bl	11.9	(13)	10.3	12.5	14.9
bl/md $\times 100$	125.2	(118)	120.0	113.0	119.0
$M^2/M^1 \times 100$	110.1	(110)	111.0	120.0	112.0

palate of M16649 and the much greater height of the buccal cusp and the greater cingulum development in M35145. In all these characters the specimens from Saudi Arabia retain, with *Proconsul* species, what is interpreted as the primitive condition for the Hominoidea⁶. They lack the synapomorphies of the *Ramapithecus-Sivapithecus* lineage of Asia⁶⁻⁸, and thus cannot be grouped taxonomically with these genera, but as they share only primitive characters with *Proconsul*, they cannot, on present evidence, be grouped with that genus either. The conformation of the mandible, when it is known, should show whether these specimens belong to the genus *Proconsul*, and until we have additional evidence we have decided not to name these specimens.

The known range of dryopithecines in Africa during the Miocene has been greatly increased by these discoveries in Saudi Arabia. This area must have been close to the migration routes between Africa and Eurasia, and it is therefore of great significance that there is no evidence linking these specimens with the contemporary species of *Sivapithecus* from Turkey⁹. The Turkish deposits at Pasalar are similar in age to the Saudi Arabian deposits, but their *Sivapithecus* species shares synapomorphies with later Miocene species of the genus from India and Pakistan⁸ that are not present in the Saudi Arabian specimens. This could indicate that the new specimens from Saudi Arabia represent a primitive branch of the Dryopithecinae that was not directly related to any of the later pongid lineages.

We thank Dr David Pilbeam for useful comments.

PETER ANDREWS
W. R. HAMILTON
P. J. WHYBROW

British Museum (Natural History),
Cromwell Road, London SW7, UK

Received 25 February; accepted 10 May 1978.

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Stability and diversity of ecological communities

MAY¹ drew an analogy between large randomly connected cybernetic networks^{2,3} and ecosystems. His analysis of stability criteria for such networks seems to contradict the traditional ecological hypothesis that ecosystem diversity contributes to functional stability⁴⁻⁷. May's analytical solution for large matrices in which each element by itself is stable showed that¹ "too rich a web connectance or too large an average interaction strength leads to instability. The larger the number of species, the more pronounced the effect". As the number of elements in the matrix increases, the transition region between stability and instability becomes increasingly narrow¹⁻³. Thus, a complex system perched near this transition region is subject to a self-generating catastrophe upon only a minor modification of system parameters. If these models are applicable to real ecosystems, they obviously have immense implications for resource management as well as ecological theory. I report here an application of May's conclusion to a real ecological system.

These cybernetic systems have three parameters¹⁻³: (1) the number of elements, n , in the interaction matrix. (2) An average interaction term, i , which characterises the average effect of each element on each other element; overall, i is

bounded at +1 and -1. (3) A connectance, c , which is the proportion of non-zero i 's in the matrix. Each element in the matrix, in isolation, will return to its equilibrium value if perturbed because of negative self-damping terms; the rate of return will be determined by the magnitude of the self-damping term. Stability is defined by a matrix in which all real parts of latent roots are negative^{1,3}. May¹ showed that very large systems are almost certainly stable if

$$i < (nc)^{-1/2} \quad (1)$$

and unstable if

$$i > (nc)^{-1/2} \quad (2)$$

Even for relatively small matrices, the analytical solution agrees well with Monte Carlo simulations^{1,8}. If equation (1) holds, any matrix element perturbed from its equilibrium will return there.

Although May's conclusion is widely cited as evidence contradicting the conventional ecological wisdom that ecosystem functional stability is facilitated by diversity, I know of no application of it to real ecological systems. I report here such an application giving evidence that more diverse plant communities are likely to be more stable, by May's criterion, than less diverse plant communities. Because these models lack much of the intrinsic structure of trophic webs^{9,10} it seems most appropriate to apply them to a single trophic level, as I do here.

A common problem in applying mathematical models to ecological systems is measuring parameters in ecological terms that are compatible with specifications of the model. I propose the following approaches to measuring n , i , and c in nature: Following May's analogy, I call the number of species, s , the number of interaction elements. I measure interaction among species by applying the point correlation coefficient¹¹, V , to nearest neighbour data¹². V has the same limits as i , being +1 if two species always occur together, and -1 if they never occur as adjacent individuals. There is ample evidence suggesting that local positive associations are caused by facilitation^{13,14} and negative associations by competition and resultant habitat segregation¹³⁻¹⁵. I estimate the average interaction term, i , as the mean value of significant V 's ($P < 0.05$ for $V = 0$). As negative associations are much more common than positive associations in the grasslands studied, I changed the sign of mean V to positive for convenience. Following rules of the cybernetic matrices^{1,3}, I define c as the proportion of all V 's that were significantly different from zero.

Data were collected in 17 grassland stands in Tanzania's Serengeti National Park during May and June 1977, a period of peak above-ground plant biomass. Species identities of the individual nearest to a random sampling point, and of its nearest neighbour, were recorded for 100 sampling points in each stand. If the primary and secondary individuals were the same species, the closest individual of another species was recorded. The range of vegetation sampled was designed to be representative of the range of grassland communities identified by clustering data from 105 stands¹⁶. V was calculated only when each species was represented by at least 10 individuals. As points rather than species were the random sampling criterion, data were corrected for sample size. That is, the number of possible associations increases as $(s^2 - s)/2$ so the probability of recording 10 individuals of all species for a standard sample size decreases with s . This was corrected for by applying the partial correlation coefficient, r_p , holding V_c/V_p constant, where V_c was the number of associations calculated based on the limit at 10 individuals and V_p was $(s^2 - s)/2$.

Inspection of equations (1) and (2) shows that stability might increase with diversity if interaction strength and connectance declined as more species were added¹. I found that both average interaction strength (Fig. 1) and connectance (Fig. 2) declined as species richness of the grassland increased. The correlation was somewhat stronger for interaction strength than for connectance.

These data provide empirical support for two conjectures about how natural communities are organised, and how this

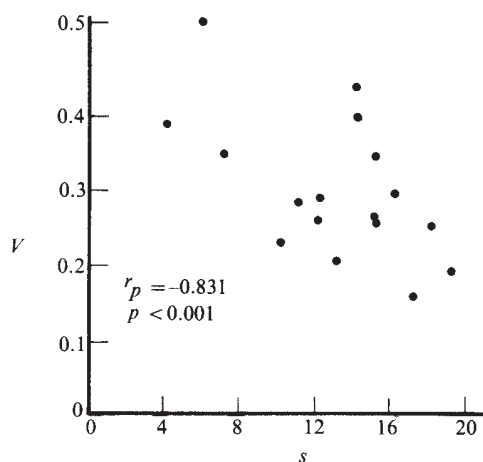
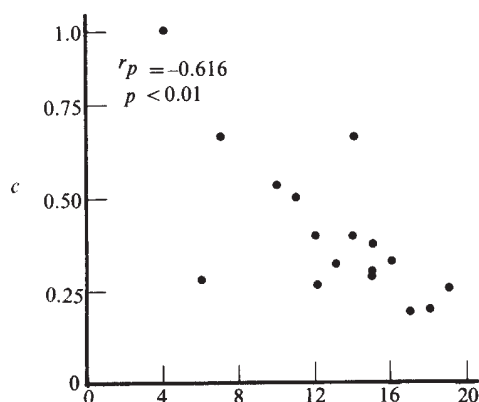


Fig. 1 relationship between average interaction strength among species and number of species in African grassland samples. r_p is the partial correlation coefficient with sample size held constant.

organisation may affect relationships between community diversity and stability. First, species-poor communities are likely to be characterised by strong interactions among the species while species interacting with many others are likely to do so weakly¹⁷. Thus, diffuse competition¹⁸ becomes more important as species diversity increases. Second, it suggests that communities are organised as blocks of species which interact among themselves but interact little with species in other blocks. May¹ remarked that such an organisation might reduce the destabilising effect of greater system complexity. I suggest that such interacting blocks of species represent guilds¹⁹ of species segregating along similar resources, and that they are only weakly connected with other guilds segregating along other gradients. Guild size may be estimated from sc . I found this product remarkably uniform for the 17 grasslands sampled: $sc = 4.7 \pm 0.7$ (0.95 interval) species per guild. For the Serengeti grassland, then, a guild may consist of remarkably uniform blocks of four to five species. Speculation about the environmental gradients organising such guilds in plant communities seems injudicious at this time. Numerous alternatives are possible.

The ratio, $i/(sc)^{-1/2}$, may be used as an instability index; if it is greater than 1, the system has a zero probability of being stable; if it is less than 1, it will be stable. Since both interaction strength and connectance declined with species richness, and $sc \approx$ constant in these samples, it is obvious that the ratio also must decrease. There was a significant negative correlation

Fig. 2 Relationship between connectance and number of species in African grassland samples.



between the index and s ($r_p = -0.594$ for $P < 0.02$ with $df = 14$). Mean value of the ratio was 0.65 ± 0.12 (0.95 interval). Only one stand, with a ratio of 1.39, fell above the critical limit of 1. In general, these grasslands would be expected to return to their equilibrium relative abundance relations if perturbed. They are stable by May's criterion¹.

Fragmentary evidence is available indicating that relative abundance relations are more stable, with environmental changes, as $i/(sc)^{-1/2}$ decreases. I reexamined in 1975 a series of four exclosures built in the early 1960s²⁰, evaluating the stability of relative abundances by the coefficient of similarity²¹ between protected and unprotected vegetation. The closer the similarity was to 1, the less species composition had changed subsequent to protection from grazing. As the ratio of interaction strength to the richness-connectance function increased in the unprotected vegetation, the less similar were species abundance relations in protected and unprotected vegetation (Table 1). Sample size is so small that statistical comparison seems inadvisable, but these data suggest a greater tendency for vegetation with weak interactions and low connectance to maintain species abundance relations when the environment changes.

In conclusion, I believe May's arguments do not contradict the traditional ecological hypothesis that ecosystem stability increases with ecosystem diversity. Rather, these arguments provide considerable fresh insight into the organisational constraints within ecosystems that may contribute to an association between diversity and stability. The occurrence of species as guilds with a relatively low number of participants, which interact weakly if at all with other guilds, may be an important component of ecosystem organisation. This is a consequence of

Table 1 Similarity of species relative abundance relations in unprotected grasslands and areas protected from grazing for over 10 yr and the instability index for unprotected areas

Similarity (C)	Instability ($i/(sc)^{-1/2}$)
0.022	0.571
0.365	0.537
0.419	0.433
0.539	0.348

a decline in connectance as diversity increases. If connectance were constant, the number of species per guild would be a linear function of the total number of species with slope = connectance. Even among those species which interact, the average strength of interaction may decline as diversity increases. Thus, more diverse ecosystems may be more stable than less diverse systems because (1) connectance declines as diversity increases, (2) species are therefore organised as relatively small guilds, and (3) interaction strength among species declines as diversity increases.

This research was supported by grants from the US NSF Ecosystem Studies Program (BMS 74-02043, DEB 74-02043-A02, and DEB 77-20360) to Syracuse University. I thank the Trustees and Director of Tanzania National Parks for the facilities of the Serengeti National Park, and Dr T. Mcharo, for inviting me to work there. I thank M. M., R. S. & E. M. McNaughton for assistance.

S. J. McNAUGHTON

Biological Research Laboratories,
Syracuse University, 130 College Place,
Syracuse, New York 13210
and
Serengeti Research Institute,
PO Seronera, via Arusha, Tanzania

Received 20 March; accepted 1 May 1978.

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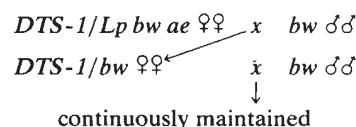
Genetic sexing technique for a mosquito sterile male release

DURING the course of an experiment to assess the efficiency of a breeding system to detect visible and lethal mutants on chromosome 3, a dominant, temperature-sensitive lethal (DTS) was discovered in the mosquito, *Culex tritaeniorhynchus* Giles, an important arbovirus vector. This DTS lethal, designated $L(3)I^{DTS}$ but here referred to as *DTS-1*, survives at 26°C but dies at 32°C during the larval stages. It was recovered after treatment with 0.005 M ethyl methanesulphonate (EMS). The isolation of a sex-linked DTS in a vector species of medical or agricultural importance may be valuable as a sexing device in a release programme involving either males sterilised directly by radiation or chemosterilants or males carrying genetic aberrations that give high semisterility.

The use of conditional lethals such as temperature sensitivity in insect control has been suggested by several investigators^{1–7}. While conditional lethals, especially temperature-sensitive ones, may be potentially useful in limiting natural populations, they can also be used as a sexing device in a sterile male release programme. The sexing of insects for release may be difficult, expensive and time consuming if it involves laborious sorting by hand, or less than 100% efficient by the use of mechanical devices. A genetic sexing method would also save space and labour as only those individuals for release are reared. In two species of medical importance, houseflies, *Musca domestica* L.⁸ and mosquitoes, *Cx tritaeniorhynchus* Giles⁹ recessive heat-sensitive lethal mutations have been isolated, after treatment with EMS, that can be used in combination with the sex-determining factors as a method of sexing adults for a sterile male release programme. However, the possibility of incorporating the genome from a wild population into the genotype of males to be released renders a dominant temperature-sensitive lethal superior to a recessive one in a scheme to produce progeny of only one sex. Recently for the mosquito, *Anopheles gambiae* Giles, a genetic sexing mechanism was produced which involved the use of a conditional lethal, diel-drin resistance, and a translocation¹⁰. Using this method, diel-drin-resistant sterile or semisterile males would be released into the field. The authors of the method suggest that this could be considered, as diel-drin resistance is already common in insect pest populations and this insecticide and its analogues are unlikely to be used as control agents because of this and because of their toxicity hazards.

In *Cx tritaeniorhynchus* ($2n = 6$) genetic systems are available to detect lethal and visible mutations on chromosomes 1^{9,11} and 2¹². A pericentric inversion, $In(3)I$, had been isolated that reduced recombination in males carrying the M^3 sex allele^{13,14} between the two linkage group III terminal markers long palpi¹⁵, *Lp*, and abnormal eye¹⁶, *ae*, from 50 map units to approximately 10 map units. Incorporating this crossover suppressor and *Lp*, M^3 , *ae*, and *bw*¹⁶ (brown eye, a recessive about 15 map units from *ae*) a mating scheme has been devised

in which a major portion of linkage group III is rendered homozygous to detect lethal and visible mutations (Fig. 1). In an experiment to assess the efficacy of this scheme an isogenic stock had been isolated which seemed to be free of recessive lethals at 26° and 32 °C. At both temperatures most of the first instar larvae usually develop into adults. Males from this stock were then treated with 0.005 M EMS to isolate visible mutants and temperature-sensitive lethals on chromosome 3. However, the control (males not treated with EMS) gave erratic results which suggested that either the original isogenic stock was no longer lethal-free or that recessive lethals had been introduced from the tester males. The latter was possible as $In(3)I$ allowed at least 10% crossing over between *Lp* and *ae*. Therefore, the entire experiment was discarded except for one line (out of the 169 investigated) which indicated the possible presence of a DTS lethal. In this line only 5 *bw* ♂♂ were found in the F_3 family reared at 32 °C while 11 *Lp* ♀♀, 16+♂♂ and 9 *bw* ♂♂ were recovered from the family reared at 26 °C. In the F_4 generation similar results were obtained at 32 °C in which the one family tested had only 18 *bw* ♂♂ while at 26 °C there were 19 *Lp* ♀♀, 16+♂♂ and 18 *bw* ♂♂. The lack of +♀♀ suggested that the *DTS-1* homozygote was itself lethal or that other recessive lethals had been induced elsewhere on the chromosome. The F_4 generation *Lp* ♀♀ carrying the *DTS-1* chromosome were then outcrossed to males from the *bw* laboratory strain for two successive generations at 26 °C:



In this maintenance breeding system all individuals with normal eyes carry the *DTS-1* mutant, which can be distinguished from the homozygous *bw* at all life stages (embryos, larvae, pupae and adults) and at all temperatures, and can be checked every generation for contamination or modification by rearing some families or part of individual families at 32 °C and 26 °C.

Using this mating scheme, we reared larvae at 26°, 28°, 30° and 32 °C to determine more precisely the lethal temperature. The results (Table 1) indicate a reduction in survival of the *DTS-1* from 26° to 32 °C with the lethal temperature between 30° and 32 °C. At 26 °C the *DTS-1* chromosome actually exhibited greater survival than the *bw*-carrying chromosome. Daily examination of the cultures reared at 32 °C indicated that the lethal phase, the development stage at which death occurs, is usually in the second and sometimes in the third instar. At 32 °C the *DTS-1* individuals remained as second instars for 2–3 d without moulting and then died while the *bw* larvae continued development with only 1 d spent at the second instar stage. By the time the *bw* larvae were in the fourth instar, most of the *DTS-1* larvae were dead.

The temperature-sensitive period, the developmental interval in which the mutant was irrevocably committed to death by the restrictive temperature, was determined tentatively by a preliminary series of reciprocal shift-up, shift-down and double

Table 1 Results of crosses *DTS-1/bw* ♀ × *bw* ♂ reared at 26 °C, 28 °C, 30 °C and 32 °C

Temperature (°C)	No. larvae/adults		% Survival larvae to adults		Survival ratio <i>DTS-1/bw</i>
	<i>DTS-1</i>	<i>bw</i>	<i>DTS-1</i>	<i>bw</i>	
26	173/133	147/94	76.9	63.9	1.20
28	157/105	151/112	66.9	74.2	0.90
30	204/27	202/125	13.2	61.9	0.21
32	275/0	294/213	0	72.4	0

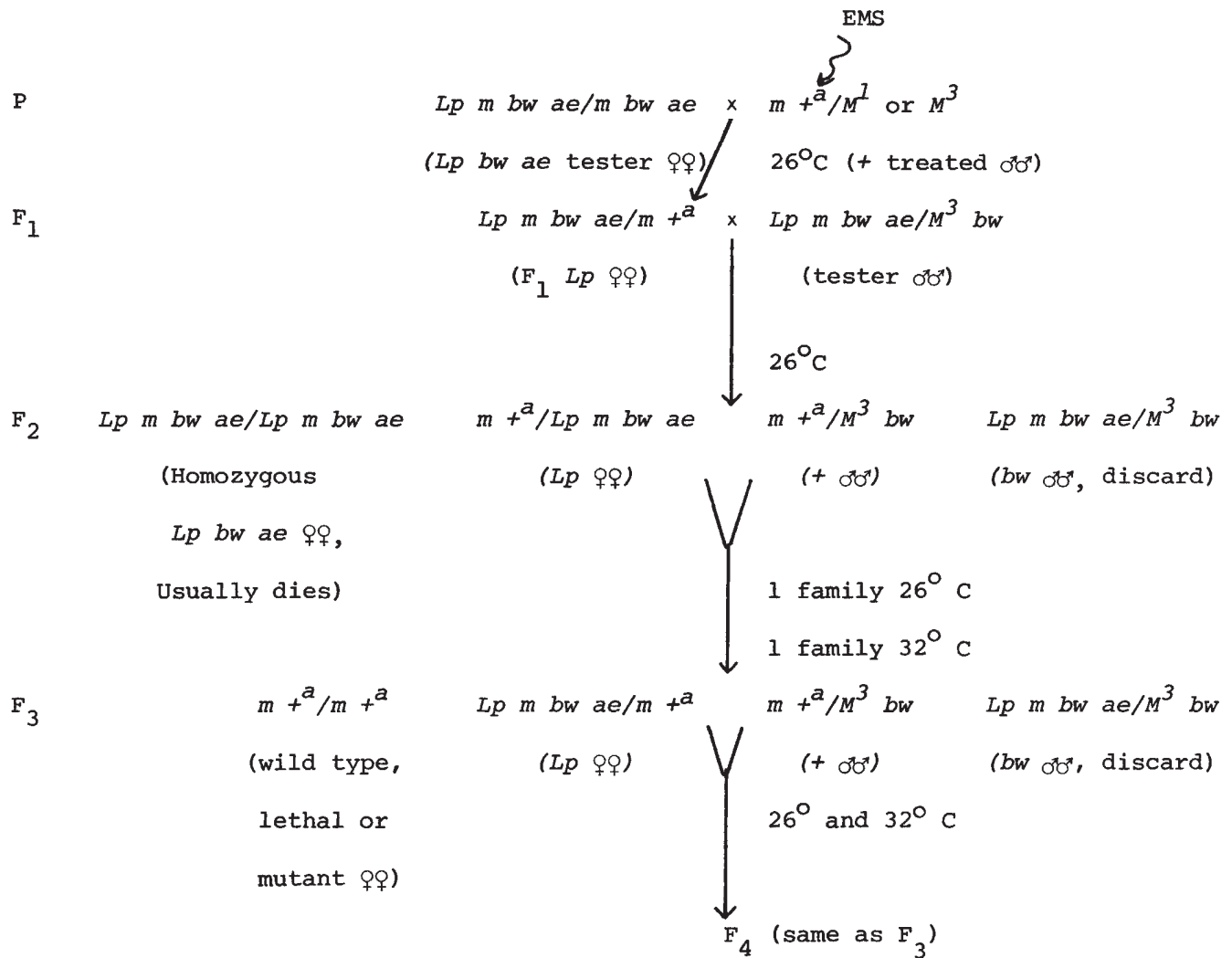


Fig. 1 Screening protocol used to recover *DTS-1* on chromosome 3, $m +^a$ denotes a single EMS-treated wild-type chromosome. The $M^3\ bw$ chromosome contains the crossover suppressor *In(3)1*, *bw* and the sex allele M^3 . Sex in this species is determined by a single pair of alleles, M and m (M for maleness) for which males are heterozygous m/M and females homozygous m/m . The sex allele M in different populations of *Cx tritaeniorhynchus* may be present on chromosome 1 (M^1) or chromosome 3 (M^3). The linear relationships among markers within a linkage group remain unaltered irrespective of whether M^1 or M^3 is present. Females behave as the homogametic sex with either M^1 or M^3 -carrying males. In this species crossover suppressors are only needed in males as linkage is generally complete in females for all three linkage groups. Females homozygous for *Lp* are phenotypically different from heterozygotes. Males carrying *Lp* either as a homozygote or heterozygote have normal male palpi.

shift experiments of 1, 2 and 3 d duration at 26 °C and 32 °C. In the egg stage, which at either 26 °C or 32 °C lasts for 24–34 h, 32 °C does not seem to affect the survival of *DTS-1*. In the larval stages the second instar period, days 2–3 out of the 7–9-d total egg-to-adult life cycle, seems to be the most sensitive period. Double shifts of 1-d duration of either 32°–26°–32 °C or the reciprocal did not affect the survival ratio of *DTS-1/bw* from that of continuous rearing at 32 °C or 26 °C respectively, while 2-d and 3-d shifts during days 2–3 greatly affected the survival of *DTS-1*.

The genetic position of *DTS-1* was determined by crosses to strains with linkage group III makers⁸. The results of the backcrosses of males heterozygous for three or four loci with the progeny reared at 32 °C are given in Table 2. The percentage hatchability was lower than expected, but 75% of the unhatched eggs appeared un-embryonated and possibly were unfertilised. Usually at 32 °C the survival of wild-type stocks is approximately 50% or better. Therefore, the approximate 25% survival for all three crosses was to be expected if half of the larvae (all *DTS-1*) died at 32 °C. The combined data for all

three crosses indicated that the *DTS-1* locus lies approximately midway between *Lp* and M^3 (5.71 and 5.69 map units respectively) and approximately 30–32 map units from *bw*. The recombination frequencies for M^3 to *bw* and *bw* to *ae* in these crosses were in reasonable agreement with those of previous studies¹³. Thus the overall gene order suggested for the five loci tested is *Lp*–*DTS-1*– M^3 –*bw*–*ae*.

With the discovery of the *DTS-1* and the M^3 loci on chromosome 3, it is now possible to produce males sterilised with radiation or chemosterilants or semi-sterile males carrying M^3 -linked chromosomal aberrations without producing adult females. Males with a crossover suppressor on the M^3 -bearing chromosome of the genotype *DTS-1* $m/+M^3$ mated to any female and reared at 32 °C are expected to produce no adult daughters:

$$\begin{array}{ccc}
 +m/+m\ (\text{♀}) \times & DTS-1\ m/+M^3\ (\text{♂}) & \\
 \downarrow & & \\
 DTS-1\ m/+m\ (\text{♀♀}) & \text{and} & +m/+M^3\ (\text{♂♂}) \\
 (\text{die at } 32^\circ\text{C}) & & (\text{survive at } 32^\circ\text{C})
 \end{array}$$

Table 2 Summary of backcrosses to elucidate the linkage relationship among *DTS-1*, *Lp*, *M³*, *bw* and *ae*

Genotype of fathers	<i>f</i>	Eggs laid	First instar larvae	Adults	% Hatch.	% Sur.	<i>Lp</i>	% Recombination			
								<i>DTS-1</i> to <i>M³</i>	<i>bw</i>	<i>M³</i> <i>bw</i> to <i>ae</i>	
<i>DTS-1</i> + +	54	7745	5800	1467	74.9	25.3			31.77		14.25
+ <i>bw ae</i>											
+ <i>DTS-1</i> + +	17	2472	2138	523	86.5	24.5	5.71*		29.64		14.53
<i>Lp</i> + <i>bw ae</i>											
+ <i>m bw ae</i>	21	1564	1309	334	83.7	25.5		5.69	30.24	24.55	19.46
<i>DTS-1 M³</i> + +											

All heterozygous males were mated to *bw ae* females and were reared at 32 °C.

f, Number of families examined. % Hatch., % hatchability: (total hatched eggs/total eggs laid) × 100. % Sur., % Survival: (total adults/total first instar larvae) × 100.

*From female progeny only, as *Lp* not expressed in males.

Moreover, the +*m*/+*m* (♀♀) can be taken from the field population of the release site, thus providing at least 1/2 of the local genome to the release males. The recessive temperature lethals isolated previously in *M. domestica*⁸ and *Cx tritaeniorhynchus*⁹ do not have this advantage as both parents of release males must be of laboratory origin.

Laboratory experiments using the above crosses have been successful in producing all male offspring. As Table 2 shows, we have isolated a line with *DTS-1* and *M³* on the same chromosome. This line normally produces approximately 95% females at 32 °C and is maintained by rearing half of the families at 26 °C and half at 32 °C and discarding families with recombinant fathers. We are attempting to isolate a crossover suppressor to link completely the *DTS-1* mutant to *M³*. With the two loci completely linked, it will then be possible to produce all-female progeny which may also be useful in release programmes and in the laboratory for the isolation of virgin females.

DTS lethal mutations have been valuable for investigating the basis of development⁴. Perhaps now they may be of value in the control of pests of medical and agricultural importance.

We thank M. Saghir, N. Hussain, M. Nasir, I. Zafar, M. Rais, M. Ali, M. Tahir, L. Chaudhari, Z. Ahmad and I. Chughtai for technical help, and the US Agency for International Development in Pakistan for assistance. This work was supported by a grant from the NIAID no. AI-10049.

RICHARD H. BAKER

RICHARD K. SAKAI

UMAIMA T. SAIFUDDIN

Pakistan Medical Research Center,
University of Maryland,
6, Birdwood Road,
Lahore, Pakistan

Received 20 February; accepted 30 May 1978.

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Haploid cell cultures of *Drosophila melanogaster*

EFFORTS to establish haploid animal cell lines have produced encouraging results only for the frog^{1,2} and cockroach³, the genetics of which are poorly understood. Attempts to use mouse teratocarcinoma, which would be more suitable in this respect, have failed⁴, perhaps because of the presence of recessive lethal genes^{4,5}. I describe here a haploid cell line established from a mutant of *Drosophila melanogaster*. Because of its simple karyotype (*n* = 4), this line will provide valuable experimental material for somatic cell geneticists.

All previous attempts to establish haploid cell lines have exploited spontaneous or artificial parthenogenesis. This phenomenon occurs in some species of *Drosophila*^{7–9} but some form of regulation always acts to yield diploid (or even triploid⁷) flies. Some diploid/haploid mosaics have been described in the mutant stocks claret and minute 1n of *D. simulans*¹⁰ and *D. melanogaster*^{11–13}, but their rare occurrence makes the use of these mutants too tedious. Nevertheless their existence was encouraging because it indicated that haploid cells are not cell lethal in *Drosophila*.

In a study of the X-linked female sterile mutants of *D. melanogaster* obtained by Gans *et al.*¹⁴, Zalokar *et al.*¹⁵ found seven that produced haploid embryos. One of them, *1182 ts*, was given to me by Gans' group. This maternal effect mutation, localised between 'vermillion' (I-33) and 'garner' (I-44), is temperature sensitive: at 18 °C the progeny of homozygous females are viable; at 25 °C the embryos die during organogenesis. Fragments of embryos derived from *1182 ts/1182 ts* females grown at 25 °C were cultured using a slightly modified version of the technique of Echaliier and Ohanessian^{6,16}. Their medium and that of Shields *et al.*¹⁷ were used in equal proportions, supplemented with 20% fetal calf serum. Numerous primary cultures were obtained, displaying the classical types of differentiated cells already described^{6,16,17}. Most of these cultures degenerated after 3–8 weeks, but six of them survived after more frequent transfer. Of these lines, three are well established and can be considered to be continuous cell lines. They are transferred every 10 d at 19 °C.

Karyotype analysis showed that our three lines have haploid metaphases of the constitution X/2/3/4 (Fig. 1). After 3 months of culture (fifth transfer) the haploid cells represented 10, 20 and 50% of the cell population in the three lines; after 6 months (12th transfer), 20, 35 and 95% and after one year (30th transfer), 50, 80 and 95%, respectively. They are not progressively supplanted by the diploid cells in the cultures, as has been observed in the past with other material.

It thus seems clear that haploid cells of *D. melanogaster* can grow *in vitro*. The cell line which contains the greatest pro-

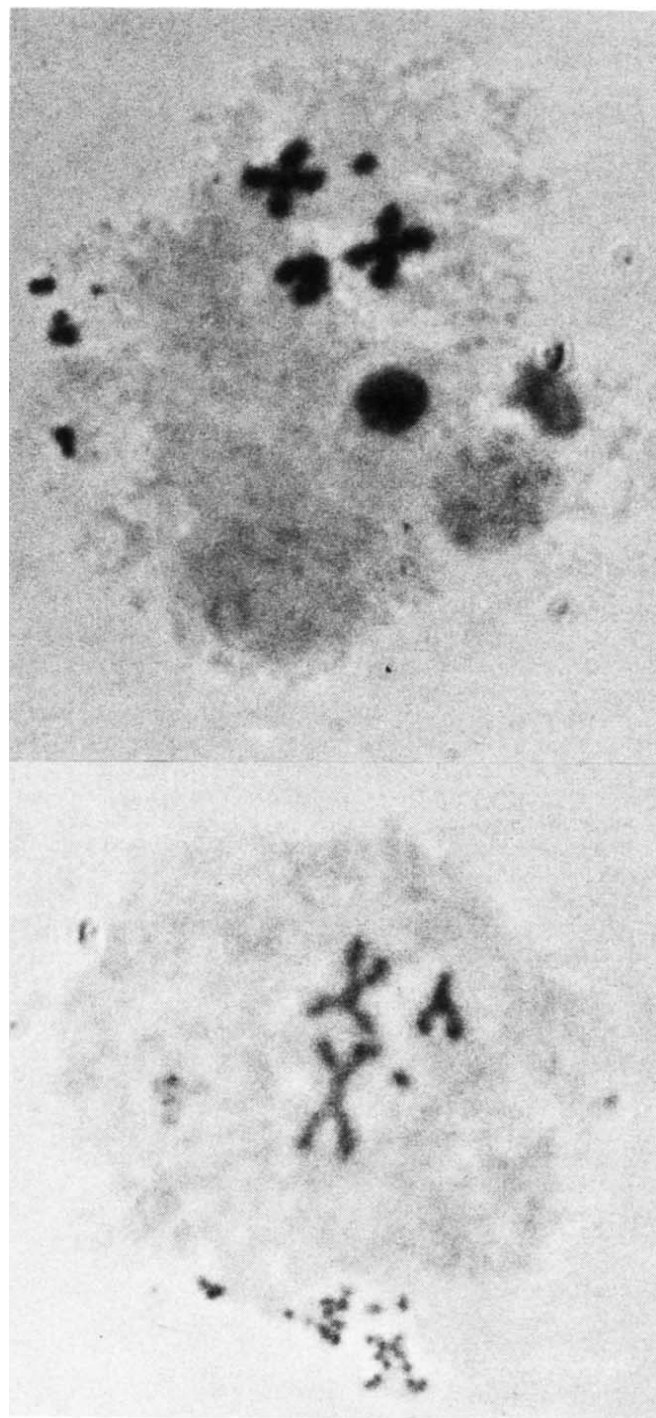


Fig. 1 Haploid metaphases of *D. melanogaster* cells cultured *in vitro*. Preparations were treated with colchicine and stained with orcein ($\times 1,000$).

portion of haploid cells is remarkably stable and can be used for genetic studies. Such material might make it possible to recover recessive mutations at a high frequency, and it will be an excellent candidate for somatic cell hybridisation.

A. DEBEC

Laboratoire de Zoologie,
Université Pierre et Marie Curie,
Batiment A 9 quai St Bernard,
75 230 Paris Cedex 05, France

Received 23 February; accepted 16 May 1978.

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Evidence for dominant mutations reducing HGPRT activity

HERITABLE variants (which we shall refer to as mutants) occur in mammalian cells with high frequency, despite the apparently recessive phenotype of the variant and despite the diploid or heteroploid nature of the cell line. For example, the frequency of 8-azaguanine (8AG)-resistant mutants¹ or of hypoxanthine-guanine phosphoribosyl transferase (HGPRT) negative mutants² does not seem to decrease as an exponential function of the increase in ploidy. Explanations for this high frequency have ranged from the presence of regulatory mutations¹, to mechanisms such as non-disjunction or somatic recombination^{2,3}. If the phenotype was due to a dominant mutation, the high frequency of mutants would be expected. We explore this hypothesis here and present two experimental criteria for establishing the presence of dominant mutations which reduce enzyme activity: we restore activity by frameshift mutations or we transfer the mutant phenotype by fusing irradiated mutants with wild-type cells.

A careful study of induced mutations leading to the loss of HGPRT activity in heteroploid L cells was carried out by Sharp *et al.*³. They used two selective protocols which yielded different sets of mutants after nitrosoguanidine (MNNG) mutagenesis. Treatment with 8AG alone yielded a high frequency, about 1 in 10^3 cells (set I), whereas treatment with 8AG + 6-thioguanine (6TG) yielded a low frequency, about 1 in 10^6 cells (set II). Mutants in set I (ref. 3, Fig. 1) usually possessed >10% enzyme activity and could be reverted by MNNG mutagenesis with very high frequency (about 3 in 10^4). Mutants in set II exhibited <2% enzyme activity and even with mutagenesis reverted to HAT resistance (that is, the ability to grow in the presence of hypoxanthine, amethopterin and thymidine) with an extremely low frequency (about 1 in 10^7). Structural gene mutants were identified in set II (refs 3 and 4).

The characteristics of set I variants could be due to some type of dominant regulatory mutation⁵ which reduces the enzymatic activity of the cell sufficiently to allow survival in 8AG but not 6TG. The mutants in set II could contain less frequent structural mutations or further regulatory mutations (or both) thereby reducing the HGPRT activity to a level that assured survival in 6TG.

We have carried out two sets of experiments that indicate the presence of dominant mutations which reduce the level of HGPRT. First, we have found that treatment with a frameshift mutagen restores full enzyme activity. Second, we have been able to transfer the reduction of HGPRT activity by fusion of irradiated HGPRT-negative cells with wild-type cells. The cell lines used are described in Table 1.

If, in the original mutation, the integrity of the structural gene has been lost, reversion by a frameshift mutagen should rarely occur. On the other hand, this treatment should restore enzyme activity if the original loss of activity was due to a dominant mutation decreasing the activity of a structural gene

Table 1 Characteristics of cell lines

Cell line	Induced by	HGPRT % wild type	Selected in	Sensitive to/Resistant to:
Mouse L (parental line) ³		100	—	8AG/HAT
L15 (set I) ³	MNNG	10–12	8AG	HAT/8AG
L16 (set I) ³	MNNG	10–12	8AG	HAT/8AG
500 (set II) ³	MNNG	<1	8AG+6TG	HAT/8AG+6TG
L _H 6*	Hycanthone	1–2	8AG	HAT/8AG+6TG
L _H 9*	Hycanthone	1–2	8AG	HAT/8AG+6TG
L _H 10*	Hycanthone	1–2	8AG	HAT/8AG+6TG
Wild-type CH ⁷		100	—	8AG/HAT

* These mutants were obtained by treating the wild type L-cell line with hycanthone as described in Table 2. Three clones with low HGPRT activity were selected for use.

which remains present. Two mutants from Capecci's set I (selected for use because they had a low, but appreciable, amount of enzyme (15% of wild-type activity)) were treated with the frameshift mutagen, hycanthone^{5,6}. (The frameshift specificity of hycanthone mutagenesis was verified using *Salmonella* tester strains from B. Ames.) In addition, both a mutant from set II and the parental L-cell line were treated with this frameshift mutagen. As can be seen in Table 2, hycanthone induced a high frequency of reversion in both set I mutants. This frequency is much higher than the frequency at which set II mutants can be induced to HAT resistance. It is also considerably higher than the frequency at which the parental cell line could be induced to mutate to the loss of HGPRT.

Several of the revertant clones were isolated and tested for HGPRT activity. In all cases the activity was between 80–100% of that of wild-type cells. The heat stability of HGPRT activity from these revertant clones could not be distinguished from the HGPRT stability in extracts from the parental cell line (data not shown). These results are consistent with removing a mutation which reduces the activity of the original HGPRT structural gene.

If dominant regulatory genes exist which reduce HGPRT activity, they should decrease the HGPRT activity of cells into which they are transferred. Goss and Harris⁸ have described the transfer of genetic markers from irradiated to non-irradiated cells. We have found that when mutant or parental cells are exposed to 6,500 rad ionising radiation less than 1 in 10⁸ cells survive (and self fusion does not restore viability). (Very small pieces of condensed chromatin are seen when these irradiated cells are blocked with colcemide.) We have fused irradiated (6,500 rad) mutant cells with wild-type recipients,

both mouse and Chinese hamster (CH), and succeeded in isolating 8AG-resistant clones with high frequency. As shown in Table 3, mutants (15 and 16) from set I were able to donate 8AG resistance with high frequency both to mouse L cells and to CH cells. This pattern of transferred resistance is in agreement with the measured enzyme activities of clones derived from such fusion transfers. In all cases these 8AG-resistant lines initially showed residual HGPRT activity. In control experiments (presented in the lower half of Table 3) we were unable to detect any increase in mutation frequency resulting from the fusion of non-irradiated wild-type cells with irradiated wild-type cells. Thus, we attribute the increased frequency of 8AG-resistant cells to transfer of a dominant trait which reduces HGPRT activity.

Mutant 500 (set II) donated resistance with high frequency to CH cells but with low frequency to mouse L cells. This difference could be due to a difference between the two strains in the stability of some factor which reduces HGPRT activity. (For example, it has been shown that HGPRT CRM proteins differ in stability⁴.) All of the lines derived from fusion were resistant only to 8AG. Resistance to 6TG was not transferred from strain 500. In a large number of different transfer experiments, we have never succeeded in transferring 6TG resistance. Thus, the set II mutant, strain L500, seems to contain a dominant gene which reduces HGPRT activity (and which can be transferred to other cells) and to have lost the HGPRT structural gene (thus, the mutant itself is resistant to 6TG, lacks residual activity and cannot be reverted with hycanthone).

After several months of growth in 8AG the fusion derivatives tended to lose their residual HGPRT activity and the activity was reduced from 15–20% of the normal wild-type

Table 2 Hycanthone-induced mutation to presence or absence of HGPRT activity

Cell line	L15	L16	L500	L(wild type)
Frequency of HAT- or 8AG- resistant mutants induced by Hycanthone*	(HAT) 1.3 × 10 ⁻³ (640)†	(HAT) 5.1 × 10 ⁻⁵ (56)	(HAT) 0/5 × 10 ⁶	(8AG) 1.3 × 10 ⁻⁵ (66)
Spontaneous mutation frequency	3 × 10 ⁻⁵ (30)	3 × 10 ⁻⁶ (3)	0/5 × 10 ⁶	0/5 × 10 ⁶
Range of enzyme activity‡ in hycanthone-induced mutant clones as percentage of parental activity	85 to 110 (22)	75 to 85 (16)	—	<1 to 18 (20)

* 10⁷ cells were plated in MEM³ + 10% fetal calf serum; after 12 h of growth they were treated with hycanthone (30 µg ml⁻¹) for 2 h. Hycanthone was removed by washing and after 3 d of growth the cells were trypsinised into MEM selective media (3 µg ml⁻¹) 8AG or HAT (hypoxanthine 680 µg ml⁻¹, sodium methotrexate (amethopterin) 90 µg ml⁻¹, thymidine 240 µg ml⁻¹, glycine 38 µg ml⁻¹). Mutant frequency was estimated from the total number of trypsinised cells which were replated.

† Number of different clones isolated or tested are given in parentheses in Tables 2, 3 and 4.

‡ Enzyme activity was tested *in vivo*³: 2–5 × 10⁴ cells plated in wells were grown in non-selective media for 18–24 h. ³H-hypoxanthine was added. After 9–12 h of incorporation the cells were washed twice with PBS, trypsinised and the cell number counted. TCA-insoluble radioactivity was measured to obtain the radioactivity per cell.

Table 3 Transfer of HGPRT suppression by fusion of irradiated mutants with non-irradiated wild-type cells*

Donor strain irradiated with 6500 rad†	Frequency of 8AG-resistant clones when fused with parental L recipient‡	Frequency of 8AG-resistant clones when fused with CH recipient (8AG-sensitive HGPRT ⁺)	Range and average of enzyme activity in fusion products	
			Average	Range of activity
15	2.3×10^{-5} (23)	1.0×10^{-4} (83)	12	10–15§ (10)
16	4.4×10^{-5} (44)	2.9×10^{-4} (352)	17	10–25§ (8)
500	2×10^{-6} (2)	4.2×10^{-4} (675)	13	5–30¶ (15)
500 ^R /CH #	Not Tested	3.9×10^{-4} (234)	13	10–40¶ (10)
Wild type L	0/10 ⁶	1×10^{-6} (1)		
Wild type CH	0/10 ⁶	1.5×10^{-6} (3)		

* The following are observations on fusions between irradiated and non-irradiated cells. Immediately after fusion, pieces of chromosome were evident in the hybrid cells but disappeared within a few days. Similarly, within the first day 90% of radioactive DNA transferred from irradiated donor cells was lost. After the selection and cloning of hybrids, the resultant clones contained a normal complement of chromosomes. No extra material either as intact chromosomes or pieces of chromosomes was detected. We do not know whether the remaining radioactivity (2%) is dispersed (reincorporated as precursor material) or present as big pieces of radioactive DNA.

† 10^6 cells in 1 ml Hank's solution were irradiated in a plastic tube on ice using a ^{137}Cs source (5,050 Ci; 190 rad min⁻¹) to deliver a total dose of 6,500 rad.

‡ 8×10^5 irradiated cells were fused with 2×10^6 non-irradiated cells using 500 HAU Sendai virus in 1 ml Hank's solution and shaken for 45 min at 37°C in a 120 cycle min⁻¹ reciprocal shaker. Fusions were plated in non-selective medium. Selection media, containing 3 µg ml⁻¹ 8AG when L cells were used as un-irradiated recipients or 9 µg ml⁻¹ when CH cells were used as un-irradiated recipients, was added 2 d later.

§ *In vivo* assay (see Table 2).

¶ *In vitro* assay: cells were extracted and enzyme assayed using a modification of the method described by Hughes *et al.*⁹

One of the 8AG-resistant clones produced by fusing irradiated 500 with CH, was used as donor.

activity to 5 or even 2%. When this happened, these clones became resistant to 6TG and could no longer be reverted by hycanthone. Despite this loss of activity, such hybrids can be used as irradiated donors and their decreased HGPRT activity again transferred to wild-type recipients. Such a secondary transfer is shown in Table 3, line 4. In this instance, an 8AG-resistant strain, 500^R/CH, derived by fusion of irradiated strain 500 with wild-type CH cells, was grown for several months in 8AG, during which time its HGPRT activity decreased to 2% and the strain became resistant to 6TG. It was irradiated and used as a donor in a second cross with a CH recipient. This transfer was as successful as the original cross using strain 500 itself. Again, resistance to 6TG was not transferred, only resistance to 8AG.

It should be difficult to transfer HGPRT activity into mutant cells which exhibit a significant reduction in HGPRT activity. Therefore, fusing 8AG-resistant mutants such as L15, 16 or 500 with irradiated wild-type cells should not give rise to

HAT-resistant clones. In contrast, cells which cannot donate 8AG resistance might be expected to accept and express genes for wild-type activity. 8AG-resistant mutants, which we produced by hycanthone treatment of wild-type cells (see Table 1), might be expected to lack dominant suppressing activity, as they were induced by a frameshift mutagen. In these cells lack of HGPRT should be a recessive trait. Thus, they should act as acceptors for HAT resistance (expression of HGPRT activity) but should not be able to donate 8AG resistance to wild-type cells. These expectations were realised in the experiments in Table 4. The 8AG-resistant hycanthone mutants became resistant to HAT after fusion with irradiated wild-type cells. Mutant 500 did not. Consistent with this result was the observation that seven other mutants from set II which we tested fell into two categories—five could transfer 8AG resistance but could not accept HAT resistance; two could become HAT-resistant but could not transfer 8AG resistance or only did so with very low frequency (data not shown).

Table 4 Transfer of HGPRT activity or of suppression of HGPRT activity

Mutant cell line	Parental L cells fused with irradiated mutant donor cells (frequency of clones selected in 8AG†)	Mutant L cells fused with irradiated donor parental L cells (frequency of clones selected in HAT†)	CH cells fused with irradiated mutant donor cells (frequency of clones selected in 8AG†)	Mutant cells fused with irradiated donor CH cells (frequency of clones selected in HAT†)
16	(44) 4.4×10^{-5}	0/10 ⁶	(352) 2.9×10^{-4}	0/10 ⁶
500	(2) 2×10^{-6}	0/10 ⁶	(675) 4.2×10^{-4}	0/10 ⁶
L _H 6*	(1) 2×10^{-6}	(162) 4.0×10^{-4}	(6) 1.2×10^{-5}	(74) 1.8×10^{-4}
L _H 9*	(1) 2×10^{-6}	(96) 2.4×10^{-4}	(3) 6×10^{-6}	(88) 2.2×10^{-4}
L _H 10*	0/5 $\times 10^5$	(123) 3.0×10^{-4}	(3) 6×10^{-6}	(122) 3.0×10^{-4}

* The spontaneous frequency of reversion to HAT resistance of the L_H lines was: L_H6 1.2×10^{-5} ; L_H9 2.3×10^{-5} ; L_H10 2×10^{-6} .

† Mouse L or mutant L-cell recipient crosses were selected in 3 µg ml⁻¹ 8AG or HAT (see Table 2). CH recipient crosses were selected in 9 µg ml⁻¹ 8AG.

These experiments provide evidence for the existence of a dominant heritable trait reducing the activity of HGPRT. This could be due to mutations in a locus (or loci) regulating the synthesis of HGPRT. Dominance could also result from mutations in the HGPRT structural gene such that its protein product will 'spoil' the activity of the HGPRT trimer⁹ as a result of negative complementation between interacting subunits. (Complementation between interacting subunits has been described by Chasin and Urlaub¹⁰.) The latter explanation requires the further assumptions that set I mutants contain more than one active HGPRT gene (that is, either duplications of the HGPRT locus or more than one active chromosome) and that transferred HGPRT genes remain active in the recipient cell. Evidence for more than one active HGPRT gene has been obtained in L cells (M. R. Capecchi, personal communication) as well as in HeLa cells¹¹.

We thank Dr M. Capecchi for L strains (wild type and mutants 15, 16, and 500); Drs M. Capecchi and M. Rechsteiner for advice on many of the experimental techniques and their comments on the final manuscript; and K. Matz and J. Hansen for assistance. Hycanthone was a gift from the Sterling Winthrop Laboratories. This research was supported by grants AI10056 and ESO1498 from the NIH and NP212 from the ACS.

AVINOAM KADOURI*
JEFFREY J. KUNCE
KARL G. LARK

Department of Biology,
University of Utah,
Salt Lake City, Utah 84112

*Present address: Department of Plant Genetics, The Weizmann Institute of Science, Rehovot, Israel.

Received 13 March; accepted 15 May 1978.

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ColE1 plasmid mobility and relaxation complex

LIKE other small plasmids, ColE1 cannot promote its own conjugal transfer. Conjugative plasmids such as F'*lac* allow its efficient mobilisation: ColE1 is nonconjugative but mobile (*Mob*⁺) or transmissible. There is now strong evidence that ColE1 transfers actively and autonomously, rather than by integration into the conjugative plasmid or by passive diffusion in the mating aggregate. Passive diffusion is unlikely as many derivatives of ColE1 are transfer-deficient (*Mob*⁻) (refs 1-4), as are some other small plasmids. Also the finding that some cells acquire ColE1 but no conjugative plasmid seems to discount cointegrate transfer; so does the observation that some transfer-deficient F'*lac* mutants can still mobilise ColE1 (ref. 5). Here we show that ColE1 conjugal mobility requires both a specific site on the plasmid DNA and sequences specifying diffusible gene products. We propose that the site is at the transfer origin, which is also where ColE1 mobility protein(s) nick ColE1 in relaxation complex⁶. Translocation of this site to another normally non-mobile plasmid, allows its mobilisation when ColE1 mobility proteins are supplied *in trans*.

A single continuous region, comprising about 30% of the ColE1 genome (0.28-0.55 on the map⁴) is necessary for mobility. Plasmid mutants in which this region is interrupted or deleted are *Mob*⁻, but their transfer can be successfully complemented by the *Mob*⁺ ColE1 or the related *Mob*⁺ ColK (ref. 3). This implies a necessity for ColE1 mobility proteins acting *in trans*. But can these mobilise any small plasmid, or are they specific to ColE1? They failed to increase the R64*drd*11-promoted mobilisation of pSC101 and pKT101 (Table 1). This suggests that ColE1 possesses a special site with which its mobility proteins must interact. We designate this site *bom* (basis of mobility): it is of course expected to be near a transfer origin.

Table 1 Mobilisation of plasmids in the presence and absence of *Mob*⁺ complementing ColE1/ColK

	Mobilisation alone (%)	Mobilisation in presence of ColE1 (%)	Mobilisation in presence of ColK (%)	
pDS1109	25	Incompatible	20	<i>nic</i> ⁺
pDS1144	<0.1	Incompatible	8	<i>nic</i> ⁺
pDS1143	<0.1	Incompatible	<0.1	
pDS1148	<0.1	Incompatible	<0.1	
pDS1145	<0.1	Incompatible	19	<i>nic</i> ⁺
Δ362	<0.1	Incompatible	28	<i>nic</i> ⁺
pGJ6	<0.1	Incompatible	<0.1	
pGJ1	<0.1	5	20	<i>nic</i> ⁺
pGJ2	<0.1	8	7	<i>nic</i> ⁺
pGJ4	<0.1	20	24	<i>nic</i> ⁺
pKT101	<0.1	<0.1	<0.1	
pSC101	0.5	0.5	0.2	

Frequency is quoted relative to the transfer frequency of the conjugative. The donor strain was AB2463 containing the I-like conjugative plasmid R64*drd*11, or in the case of pSC101, W3110 containing the I-like R144*drd*3. The recipient was CSH27 *rpsE*. pKT101 is a Km^r derivative of the naturally occurring ColD (K. Timmis, personal communication).

Deleting the *bom* site/transfer origin should render a plasmid *Mob*⁻, and non-transmissible even when *Mob*⁺ gene products are supplied. Nine deletion mutants of a *Mob*⁺ ColE1::TnI plasmid (pDS1109²) were obtained after *RHae*II restriction/ligation *in vitro*. They were screened for transmissibility in the presence and absence of a complementing plasmid. Five lacked the 1.1 kilobase *Hae*II-C fragment^{6,7} to the left of the origin, and only these five were *bom*⁻; they could not be mobilised by complementation. Maps and mobilisation data for two such *bom*⁻ (pDS1143 and pDS1148) and two *bom*⁺ (pDS1144 and pDS1145) plasmids are given in Fig. 1 and Table 1. The 1.1 kilobase fragment was deduced to contain *bom*. Note that it also contains *nic* (the site at which relaxation complex nicks ColE1⁶) and a part of the region encoding mobility proteins.

nic is therefore dispensable for plasmid maintenance: this argues against a role for it in vegetative replication. And since *Mob*⁻ mutants lack relaxation complex², this argues for a role in mobilisation. Particularly, it suggests that *bom* is at *nic*, and that relaxation complex is comprised of mobility proteins. Relaxation complex not only nicks the ColE1 DNA at a specific site: one of the constituent proteins becomes covalently attached to the 5' terminus of the nicked strand⁸ (see Table 1). In this respect it is similar to *cisA*, the site-specific nickase specified by cistron A of bacteriophage ΦX174 (ref. 9). We propose a model for ColE1 mobilisation based on a close analogy with ΦX174.

cisA nicking of ΦX174 duplex DNA is followed by rolling circle replication which displaces the nicked parental strand, starting at the 5' end. But *cisA*, and the attached 5' end, remain locked in the replication fork. After a round of replication,

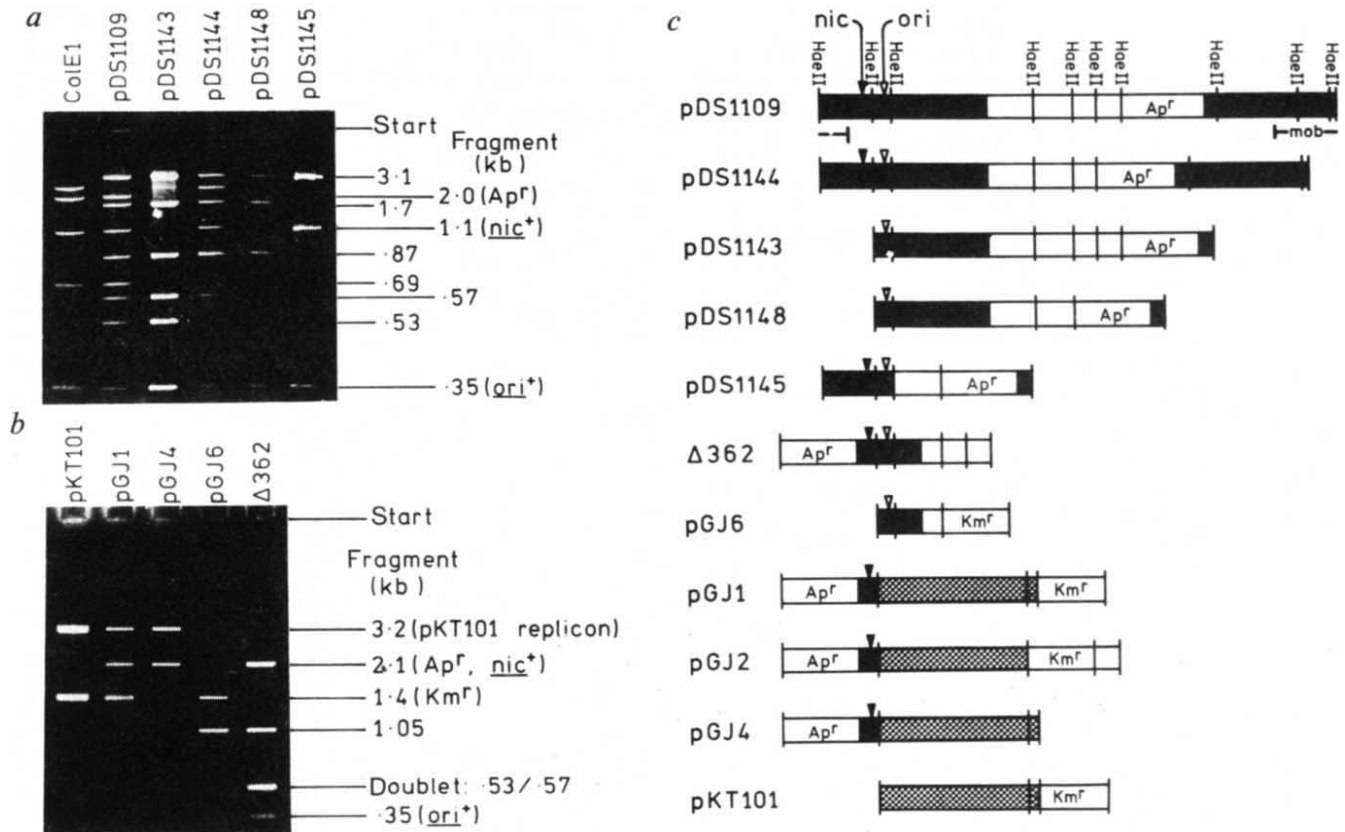


Fig. 1 Restriction endonuclease *HaeII* mapping of the ColE1 plasmids discussed here, and the vector pKT101. pKT101 is a Km^r derivative of ColD (K. Timmis, personal communication). **a**, *RHaeII* digests of pDS1109 and derivatives; 5% polyacrylamide gel, 120 V (ref. 6). **b**, *RHaeII* digests of Δ362 and derivatives; 1.4% agarose gel, 25 V (ref. 7). **c**, Scale maps deduced from complete and partial (not shown) *RHaeII* digests. The orientation of each *HaeII* fragment is not known and has been arbitrarily represented. *nic* (▼) is the ColE1 site of relaxation complex nicking. *ori* (V) is the replicative origin of ColE1. Solid bars represent ColE1 sequences. Open bars represent Tn1/3 sequences and the Km^r *HaeII* fragment of pKT101. Stippled bars represent the other vector (pKT101) sequences. Ap^r, Km^r the determinants for ampicillin and kanamycin resistance, respectively.

cisA nicks again at the origin of replication, and ligates the displaced single strand to give a viral circle. For ColE1, we suggest that after relaxation, a parental strand is displaced into the recipient, starting with the 5' end with its attached mobility (relaxation complex) protein. After transfer of a complete genome, and perhaps some complementary strand synthesis, the mobility protein nicks again at the same site, and ligates the transferred DNA into a circle (Fig. 2). This readily explains the rapid recircularisation of the transferred DNA in the recipient (replication) without the need for host recombination functions. The genetically defined site at which the mobility proteins act (*bom*) should therefore correspond to the physically defined site of relaxation complex nicking (*nic*), and this site should function as a transfer origin.

One test of these ideas was to isolate *nic* by translocating it to another replicon, and then to test for mobility and relaxation. We chose pKT101 as a vector unaffected by ColE1 *in trans*. The 'donor' of *nic* was RSF1050-Δ362: in this plasmid, most of the original 1.1 kilobase ColE1 *HaeII* fragment has been deleted, leaving *nic* linked to the right-hand end of Tn3. The translocation was achieved by mixing the DNA of pKT101 and Δ362, and performing an *in vitro RHaeII* restriction/ligation. Figure 1 gives the maps of Δ362 and three pKT101 derivatives (pGJ1, pGJ2 and pGJ4) carrying a translocated *nic* no longer linked to the ColE1 vegetative origin. A deletant of Δ362 which has lost *nic* (pGJ6) is also shown.

Attempting mobilisation of these plasmids gave results entirely consistent with our hypothesis (Table 1). Basically,

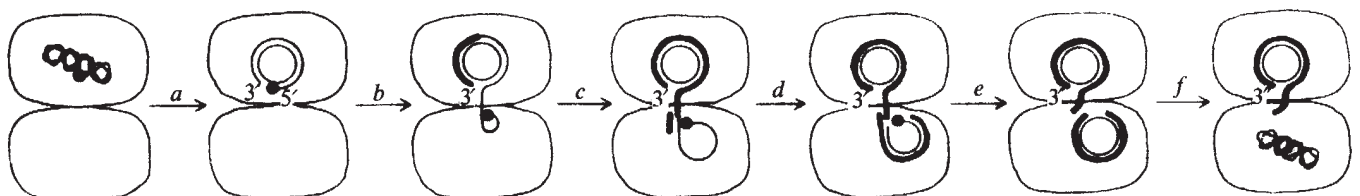


Fig. 2 Our model for ColE1 mobilisation. Heavy lines represent newly synthesised DNA. **a** Relaxation of the supercoiled DNA-protein complex in the donor; one *Mob* protein becomes covalently attached to the 5' end of the nicked strand. **b**, Transfer of the *Mob* protein and 5' end; rolling-circle DNA synthesis in the donor. **c**, Transfer of one whole genome is completed; complementary strand synthesis is initiated in the recipient. **d**, A second nicking of duplex DNA by the *Mob* protein at the transferred *nic* sequence; complementary strand synthesis proceeds in the recipient. **e**, Ligation by the *Mob* protein to give a circle in the recipient (replication). **f**, Complementary strand synthesis is completed, and supercoiling occurs.

Table 2 Relaxation of *nic⁺bom⁺* plasmid pGJ4 and the control *nic⁻bom⁻* vector pKT101 in the presence and absence of complementing ColE1

% Relaxation of	Alone	With ColE1 present	
pGJ4	18–23	47–55	
pKT101	15–19	11–17	
% relaxation of	Alone	With pGJ4 present	With pKT101 present
ColE1	83–95	63–69	70–78

The ranges of values obtained in four tests are given. The degree of relaxation of ColE1 itself in these tests is included for reference. DS410 was the host strain².

everything with *nic* could be mobilised, but pGJ6 and pKT101—both minus *nic*—could not. So *bom* must be close to *nic*, between map coordinates 0.21–0.26 (ref. 4).

The smallest plasmid with a cloned *nic*, and that capable of the highest mobilisation frequency, is pGJ4. We sought to demonstrate that its DNA could be relaxed by a complementing *Mob⁺* plasmid just as mobilisation can be complemented. Plasmid DNA was isolated from cells containing the relevant plasmids, grown in conditions appropriate for obtaining relaxation complex¹¹. Supercoiled and relaxed (open circular) plasmid species were separated on agarose gels, and their relative quantities measured by a microdensitometer. From the numbers in Table 2, pGJ4 is clearly complemented to relax.

So, as our model predicts, we have been unable to separate *bom* from *nic*, though both have proved to be separable from *ori*. Because of their potential independence, it is curious that *nic* and *ori* are found so close together in ColE1, a situation which also occurs in R6K (ref. 12). This was considered evidence that relaxation complex was involved in vegetative replication. We cannot rule this out for plasmids other than ColE1, especially as replication involves DNA gyrase, which can also act as a site-specific nickase–ligase^{13,14}. The value of our observations is that we can now embark on the biochemical analysis of ColE1 mobilisation, based on properties of ColE1 relaxation complex. We can also suggest that any 'safe' cloning vector should lack a *bom* site.

G.J.W. and A.J.T. were supported by MRC and SRC research studentships, respectively.

GARETH J. WARREN
ANDREA J. TWIGG
DAVID J. SHERRATT

School of Biological Sciences,
University of Sussex,
Brighton, UK

Received 5 April; accepted 26 May 1978.

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Pharmacological control of prostaglandin and thromboxane release from macrophages

MACROPHAGES have a key role in mediating inflammatory reactions¹. They appear in large numbers in inflamed tissue, and on contact with irritant particles release comparatively large quantities of inflammatory mediators, including prostaglandins (PGs)². Although it is known that anti-inflammatory drugs can inhibit synthesis and consequently release of PGs^{2,3}, it is not clear which cellular events lead to PG formation in macrophages during phagocytosis. We report here our use of various drugs to modulate PG release and phagocytosis and our attempts to correlate the drug effects on these two parameters with morphological changes of macrophages as visualised by transmission electron microscopy. We found that PG release in macrophages is concomitant with the formation of large vacuoles. This observation suggests that the formation of these vacuoles may be a crucial event for the formation and release of PGs from macrophages.

Table 1 shows that phagocytosis of antibody-coated erythrocytes (EA) triggered release of mainly immunoactive PGE₂ but also of measurable amounts of thromboxane B₂ (TXB₂) (and PGF_{2α}, not shown). Of the compounds tested which modulate PG and TX release from phagocytosing mouse peritoneal macrophages *in vitro*, we found cytochalasin B and 2-deoxyglucose especially interesting. 2-Deoxyglucose inhibited both phagocytosis and PGE₂/TXB₂ release, whereas cytochalasin B, in contrast, inhibited phagocytosis but at the same time significantly increased PGE₂ and TXB₂ release. As expected, indomethacin almost completely inhibited PGE₂ and TXB₂ release⁴ although phagocytosis was not measurably altered. Also, when indomethacin (10⁻⁶M) was added with cytochalasin B (10⁻⁶M) it still almost completely inhibited PGE₂ and TXB₂ release. All the drugs used influenced the release of PGE₂ and TXB₂ in a parallel manner, suggesting that phagocytosis and these drugs modify PGE₂ and TXB₂ release by influencing one or several steps in the biosynthetic pathway to PGG₂ or PGH₂, the precursors of both PGs and TXB₂. This is important as there is evidence that some drugs might increase PG synthesis at the expense of decreased TX production^{5,6}. As phagocytes are able to produce PGs⁷ and thromboxanes², this possibility had to be excluded. One may now speculate that during phagocytosis cell membranes become internalised and membrane phospholipids come in contact with the phospholipase–cyclooxygenase system in the cell interior, thus becoming the substrate for the synthesis of PGs and TXs. If this concept is correct, drugs which interfere with phagocytosis and, consequently, with (membrane) internalisation, should inhibit PG release, and drugs which induce phagocytosis-like membrane internalisation should increase PG release. To evaluate this concept we obtained transmission electron microscopical pictures of macrophages exposed to antibody-coated chicken erythrocytes (EA) alone or in the presence of either cytochalasin B or 2-deoxyglucose. The results show that phagocytosis alone induces the formation of large vacuoles and channel-like structures (Fig. 1). This effect on cell morphology is considerably increased when cytochalasin B is added although this compound inhibits phagocytosis (Fig. 1b). On the other hand, 2-deoxyglucose inhibits phagocytosis-induced morphological changes parallel to the inhibition of PG (TX) release (Fig. 1c) (the corresponding morphometric data will be published elsewhere (H. K. and K. B.)).

To test further the observed correlation between the formation of vacuoles and PG release we used 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and concanavalin A (con A). TPA was recently reported to be a potent trigger of PG release from

methylcholanthrene-transformed canine kidney cells⁸ and is known for its irritating and cocarcinogenic potency⁹. We found that TPA induced a significant increase of PGE₂ and TXB₂ release from resting macrophages and concomitantly caused the formation of many vacuoles (Fig. 2). These vacuoles and PG release are not a sign of cell death as macrophages survived TPA treatment (10⁻⁸M in the culture medium) for several days without measurable loss in viability. Con A, on the other hand, is known to induce pinocytotic vesicles in macrophages. These structures seem to be 'empty' under the electron microscope, and they do not fuse with other cell organelles, in contrast to phagocytic vacuoles¹⁰. In good agreement with these observations, we did not observe an increased release of PGs from macrophages incubated for up to four hours with con A (data not shown).

In macrophages some types of vacuoles, in contrast to pinocytotic vesicles¹⁰, are believed to result from the fusion of parts of the Golgi system, the endoplasmic reticulum, lysosomes and possibly other constituents of the cell interior¹¹. Our results indicate that the formation of vacuoles in macrophages coincides with PG formation. An obvious interpretation of our results is that the fusion of different cell organelles will bring together membranes containing arachidonic acid and phospholipases, for example, from lysosomes¹², and cyclo-oxygenase, which is probably embedded in the endoplasmic reticulum^{13,14}. The increased contact of the enzymes with their respective substrates will then result in increased formation of PGs. Phagocytosis is apparently one possible trigger leading to the formation of such vacuoles; consequently, it induces PG release. Inhibition of phagocytosis and of formation of vacuoles by 2-deoxyglucose, on the other hand, abolishes this stimulus for PG synthesis and release. In contrast, cytochalasin B enhances the formation of both vacuoles and PGs although it

inhibits phagocytosis. Similar functional and morphological changes can be induced by cytochalasin B in polymorphonuclear leukocytes¹⁵. Allison^{16,17} has suggested that these effects of cytochalasin B result from dissolution of microfilaments which normally form a network beneath the cell surface preventing contacts and fusion between the cell membrane and the membrane of cell organelles such as lysosomes. This interpretation would also explain the increased PG and TX synthesis by cytochalasin B-treated macrophages. In nonphagocytosing fibroblasts, however, cytochalasin B does not cause similar morphological changes¹⁸. Consequently, PG production by such cells would not be expected to be stimulated by this drug. Indeed, cytochalasin B has been observed to reduce PG release from transformed fibroblasts¹⁹. Other compounds like TPA can obviously induce fusions of cell organelles in macrophages in the absence of phagocytosis (Fig. 2). Although it is not clear where TPA acts in macrophages, it causes striking perturbation of the organisation of the cell interior (Fig. 2). The formation of vacuoles which can be observed within 10 min of addition of TPA is again accompanied by the increased formation of PGE₂ and TXB₂ (see legend to Fig. 2). In confluent layers of 3T3 cells, on the other hand, TPA induces neither vacuole formation²⁰ nor an increase of PG release above about 1 ng ml⁻¹ over 16 h (H. K. & K. B., unpublished).

In conclusion, we feel that there is a striking coincidence of PG release and the formation of vacuoles in macrophages. We believe that this observation may be useful in understanding why these cells are particularly effective in producing PGs. Also, it indicates which cellular mechanism(s) lead to PG formation, in, for example, inflamed tissue. Furthermore, our results suggest that the formation of vacuoles may have an important function in macrophages, namely formation and

Table 1 Effects of cytochalasin B, 2-deoxyglucose and indomethacin on prostaglandin and thromboxane release from mouse peritoneal macrophages

	5 × 10 ⁶ EA per ml						5 × 10 ⁷ EA per ml					
	Phagocytosis* (E per 100 macro- phages)	P‡	PGE ₂ † (ng ml ⁻¹)	P‡	TXB ₂ † (ng ml ⁻¹)	P‡	Phagocytosis* (E per 100 macro- phages)	P‡	PGE ₂ † (ng ml ⁻¹)	P‡	TXB ₂ † (ng ml ⁻¹)	P‡
Saline + A, no E	—	—	2.3 ± 0.3	—	0.25 ± 0.06	—	—	—	2.8 ± 0.3	—	0.28 ± 0.07	—
Saline + EA	87 ± 9	—	6.9 ± 0.5	<0.01	0.58 ± 0.17	<0.01	201 ± 33	—	45.0 ± 6.7	<0.01	1.76 ± 0.27	<0.05
Saline containing 0.5% DMSO + EA	81 ± 6	—	7.5 ± 1.0	<0.05	0.39 ± 0.06	<0.01	189 ± 22	—	43.8 ± 4.8	<0.01	1.20 ± 0.23	<0.05
2-Deoxyglucose 5 × 10 ⁻² M	53 ± 13	<0.01	2.9 ± 0.5	<0.01	0.34 ± 0.06	<0.05	62 ± 28	<0.01	1.6 ± 0.4	<0.01	0.03	<0.01
2-Deoxyglucose 5 × 10 ⁻³ M	78 ± 12	>0.05	6.9 ± 1.2	>0.05	0.41 ± 0.09	>0.05	NT	NT	NT	NT	NT	NT
Indomethacin 10 ⁻⁶ M	83 ± 11	>0.05	2.4 ± 0.5	<0.01	0.08 ± 0.05	<0.01	172 ± 26	>0.05	1.4 ± 0.3	<0.01	0.05 ± 0.01	<0.01
Indomethacin 10 ⁻⁷ M	80 ± 8	>0.05	3.3 ± 0.6	<0.01	0.13 ± 0.06	<0.01	NT	NT	NT	NT	NT	NT
Cytochalasin B 10 ⁻⁶ M	47 ± 9	<0.01	13.5 ± 0.7	<0.01	0.83 ± 0.04	<0.01	79 ± 14	<0.01	57.9 ± 6.4	<0.01	2.00 ± 0.61	<0.05
Cytochalasin B 10 ⁻⁷ M	89 ± 11	>0.05	9.5 ± 1.8	<0.01	0.40 ± 0.06	>0.05	210 ± 31	>0.05	44.6 ± 3.6	>0.05	1.44 ± 0.25	>0.05

Unstimulated macrophages of NMRI-mice were collected from the peritoneal cavity: 2 × 10⁶ cells were incubated overnight in 35-mm Petri dishes in 2 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS). Then, nonadherent cells were washed off and fresh DMEM + 5% FCS was added with chicken erythrocytes (E), drugs or solvent, and the reaction mixtures incubated further. In a first series of experiments, 10⁷E previously incubated in a subagglutinating concentration of mouse anti-chicken erythrocyte antibody (A) and then washed were added to the cell cultures and samples collected and centrifuged every hour. PGE₂ and TXB₂ content in unextracted culture medium was determined by radioimmunoassays^{20,21}. Plateau concentrations were reached after one hour. None of the drugs used interfered nonspecifically with the radioimmunoassays. Rapid metabolism did not take place in these cultures, for less than 5% of added ³H-PGE₂ was decomposed within four hours as judged by TLC-analysis. Evidence provided by others indicates that PGs found in the supernatants of macrophage culture after phagocytosis represent *de novo* synthesis²². In a second series drugs were added one hour before the addition of EA either in saline (2-deoxyglucose only) or in saline containing dimethylsulphoxide (DMSO), and supernatants were collected two hours after addition of EA. When tested by dye exclusion the number of dead cells was not increased by the drugs above controls. The results given are means ± s.d. (n = 5). All the experiments reported were repeated four times, and gave identical results with respect to drug effects.

* For determination of phagocytosis cell layers were incubated with NH₄Cl (ref. 23) fixed with 2.5% formaldehyde in Ringer solution for microscopic evaluation.

† TXB₂ concentration in media supplemented with 5% serum is 2.29 ± 0.29 ng per ml (mean ± s.e., n = 7). Therefore, all samples analysed in the TXB₂-radioimmunoassay were compared with appropriate binding controls, that is samples containing the same volume of fresh incubation medium. All values given are corrected for the TXB₂ concentration in the incubation medium. To confirm the identity of TXB₂ and PGs measured, macrophages were incubated in serum-free medium with 10⁷ EA for two hours. The media were acidified to pH 3.5, extracted into diethylether and chromatographed on thin layer plates (diethylether: methanol: acetic acid 90:1:2) with authentic PGE₂, PGF_{2α}, PGD₂ and TXB₂. The coincident zones were eluted and assayed with the respective specific antibodies. In these conditions similar amounts of PGE₂, PGF_{2α} and TXB₂ were found to those in unextracted media. PGD₂ was not detected.

‡ P values (Student's *t*-test) were calculated comparing phagocytosing macrophages (+EA) with nonphagocytosing cells (no E). Drug effects on phagocytosing cells were evaluated by comparison with the corresponding solvent control groups. NT, not tested.

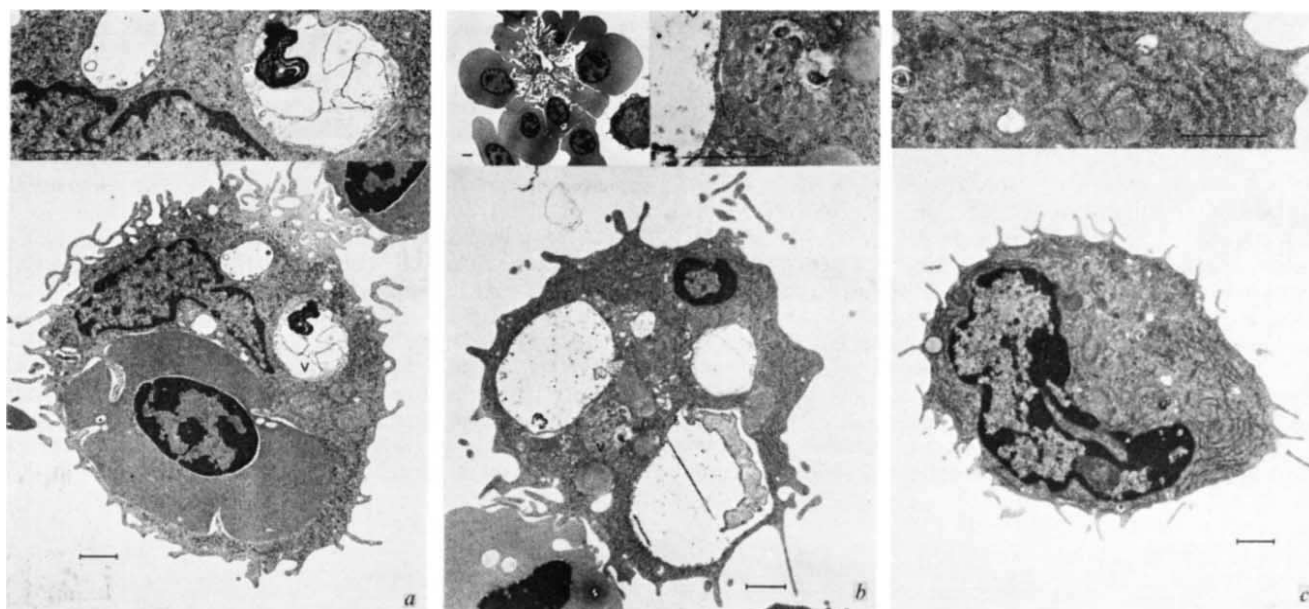
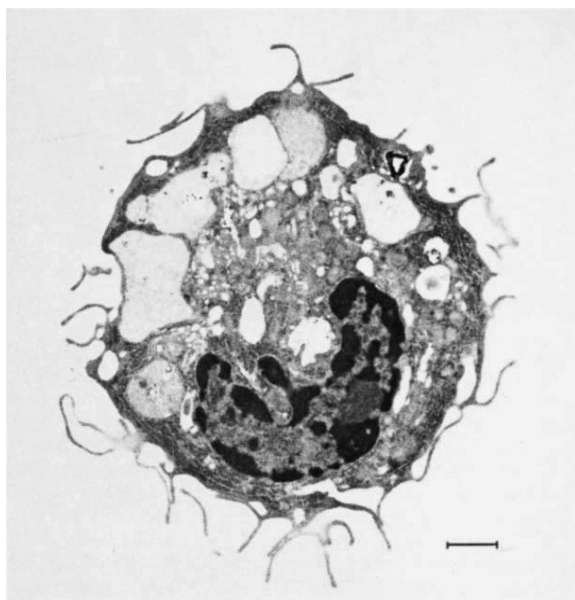


Fig. 1 Effects of cytochalasin B (b) and 2-deoxyglucose (c) on phagocytosing macrophages compared with non-drug treated controls (a). Macrophages were obtained and handled as described in the legend to Table 1 except that they were grown on plastic coverslips in Petri dishes and that incubation was stopped 30 min after EA were added, that is, 90 min after changing the medium and adding the drugs or the solvents. At that time the culture fluids were assayed for PGE₂ and TXB₂ content. (The values (mean $\pm$ s.d., ng per ml, six cultures each) were: a, PGE₂, 1.6 ± 0.5 ; TXB₂, 0.11 ± 0.06 . b, PGE₂, 2.9 ± 0.2 ; TXB₂, 0.75 ± 0.14 . c, PGE₂, 1.0 ± 0.1 ; TXB₂, 0.22 ± 0.10 .) The macrophages were fixed on the coverslips in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, containing 5% sucrose, for 60 min, washed and postfixed for 60 min in veronal acetate buffered 1% osmium tetroxide. After dehydration the cells were embedded on the coverslips in Epon 812 and the coverslips removed after polymerisation. Ultrathin sections were stained with uranyl acetate and lead citrate and photographed with a Zeiss 10 electron microscope. The pictures show the following details: a, macrophage phagocytosing an antibody-coated erythrocyte. The inset shows the one vacuole (V) of this cell at higher magnification. b, Effects of cytochalasin B on macrophages exposed to EA. Left hand inset, contact with several EA leads to degranulation and channel formation. Right hand inset, higher magnification of a large V located between two large channels (emptied V?). c, Effect of 2-deoxyglucose on macrophages exposed to EA. The cell interior is apparently well preserved; no V are detectable (inset). Bar = 1 μ m.

Fig. 2 Formation of vacuoles (V) in macrophages following exposure to 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Macrophages were obtained and handled as described in the legend of Fig. 1. Instead of antibody-coated erythrocytes, TPA in DMSO (final concentrations in the culture fluid 10^{-8} M TPA, and 0.5% DMSO) was added 60 min after changing the medium. The supernatants were assayed for PGE₂ and TXB₂ content 30 min after addition of TPA. (The values were (mean $\pm$ s.d., ng ml⁻¹): controls: PGE₂, 2.2 ± 0.3 ; TXB₂, 0.11 ± 0.06 . TPA: PGE₂, 8.7 ± 1.1 ; TXB₂, 1.31 ± 0.32 .) A representative electron microscopical picture of a macrophage exposed to TPA for 30 min is given, showing large vacuoles. Bar = 1 μ m.



release of PGs, of interest because little is known about the origin and function of these cell organelles¹¹.

This work was supported by a grant from the Swiss NSF. We thank Professor L. Levine for helpful discussions and Professor E. Hecker for providing TPA.

K. BRUNE
M. GLATT
H. KÄLIN

Department of Pharmacology,
Biocenter, Klingelbergstrasse 70,
CH-4056 Basel, Switzerland

B. A. PESKAR

Department of Pharmacology,
University of Freiburg,
Katharinenstrasse 29, D-78 Freiburg, FRG

Received 7 February; accepted 17 May 1978.

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Localised insertion of new cell wall in *Bacillus subtilis*

THE geometry of the growth of the bacterial cell surface has been studied by following the segregation of differentially labelled old and new areas of the envelope¹⁻¹⁰. Results obtained with rod-shaped bacteria led to apparently contradictory interpretations⁵. The incorporation of labelled precursors into the cell wall of Gram-positive bacilli has failed to reveal segregation and favours random insertion of new wall material^{8,9}. However, dispersion of surface components after incorporation could mask a localised pattern of insertion^{5,10,11}. To try to distinguish between the two possible modes of insertion we have investigated the pattern of cell wall segre-

gation in a mutant of *Bacillus subtilis*, which has a markedly slower cell wall turnover, so that it is possible to follow for much longer than with strains having normal, faster cell wall turnover¹². We report here a clear segregation of old and new wall, indicating localised insertion of material.

B. subtilis 168 *trp* *thy* and a derived mutant, Ni15, grew on a glucose-casein hydrolysate medium at 44 °C with a generation time of about 20 min. Ni15 grew in chains of eight to 16 bacteria while the parent grew in pairs and chains of four to eight bacteria. The cell wall was labelled specifically with *N*-acetyl-D-[1-³H]glucosamine (³H-GlcNac)¹¹. Septa were stained with crystal violet and the segregation of label was observed by standard light microscope autoradiography¹⁴ (see legends for details).

Five generations after chasing a continuously labelled culture of Ni15 dense clusters of grains could be seen exclusively at the ends of chains (Fig. 1e). The clusters corresponded both in number (about 1 per 15 bacteria) and in the proportion of

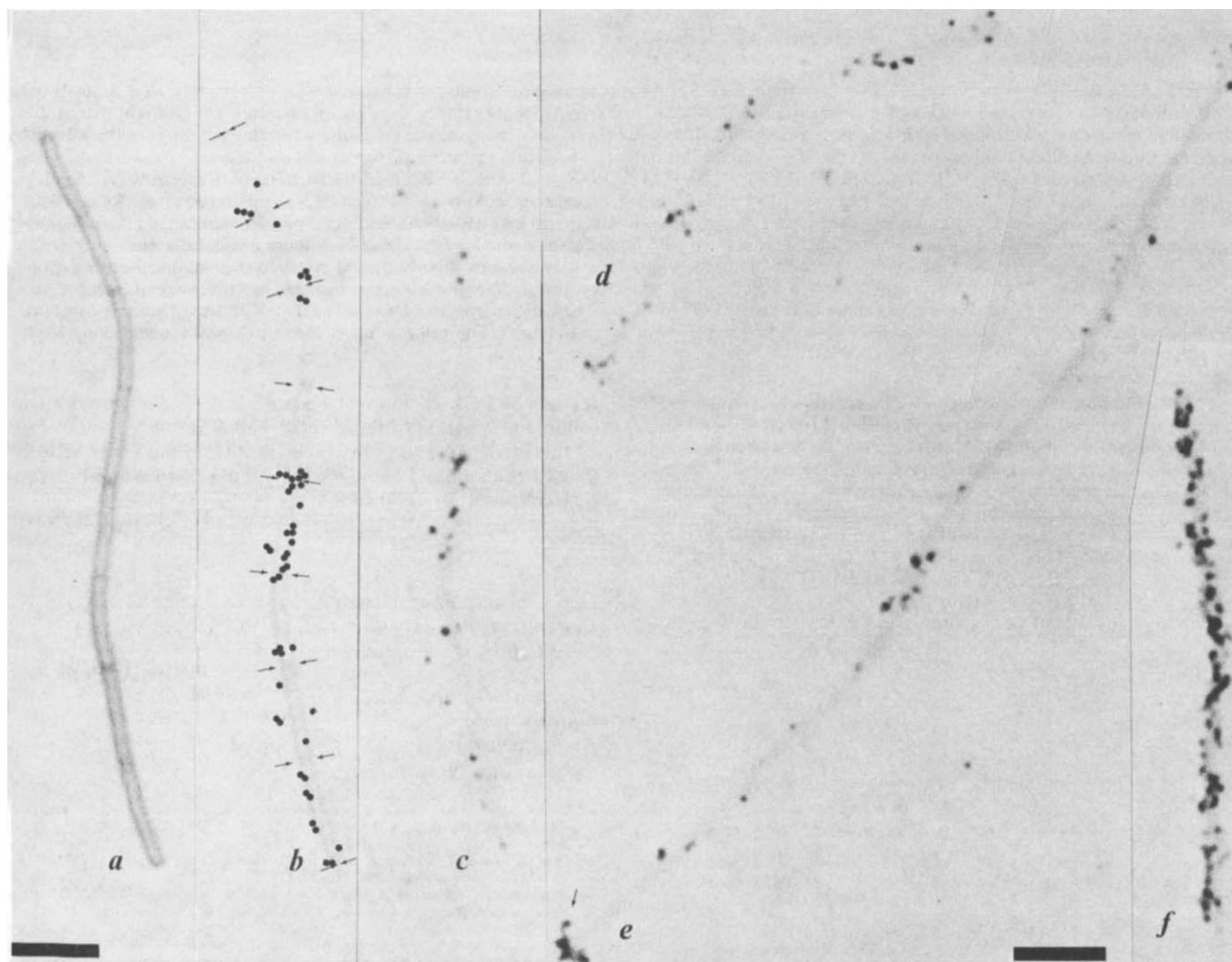


Fig. 1 Chase of continuously labelled cells. Ni15 grown for five generations at 44 °C was inoculated into medium¹¹ containing ³H-GlcNac at an A_{540} of 0.003 and grown for 5 to 6 generations. Incorporation was stopped at an A_{540} of about 0.1 by filtration and washing with warmed unlabelled medium. Cells were resuspended in chase medium at an A_{540} of 0.007 and refiltered after 0, 1.5, 3 or 5 generations. Cells were treated with trichloroacetic acid (5%) for 1 min, washed with water and stained with 0.02% crystal violet for identification of septa, *a*, After drying at 60 °C, slides were dipped in photographic emulsion (Ilford L4) and autoradiographs were prepared by standard procedures¹⁴. Chains, previously photographed for septa (*a*) were located on the developed slide and re-photographed (*c*). The position of septa was traced on to transparent paper and, when the chain had been located for the third time, individual grains, identified microscopically, were also marked on the transparent paper (*b*). After determination of the mid-point between neighbouring septa, grains per half-cell were counted and their distribution was compared with that of Poisson (Table 1). Chains are shown at 0 (*f*), and five generations (*c*, *d* and *e*). The arrow in (*e*) indicates a septal cluster. The bar is equivalent to 5 μ m. To give comparable numbers of grains per cell, the specific activity during incorporation was different for each culture. Media for 0, 1.5, 3 and 5 generation samples contained 0.065, 0.17, 0.45 and 1.375 Ci per mmol, respectively. Total GlcNac was 60 μ M.

grains (13% of the total) to the septa already formed at the time of chasing. This clear segregation indicates that wall material incorporated into septa does not become dispersed over the cylindrical part of the rod during subsequent growth.

Five generations after chasing, grains which were not in septal clusters were clearly not distributed randomly (Fig. 1 c-e). Thus there had been segregation of most of the label (~87% of the total), not merely that in the septa. These observations were substantiated by statistical analysis of grain distribution (Table 1, Fig. 1). Just after chasing (0 generation) a Poisson distribution was found. After 1.5 and 3 generations there was a lower probability of finding a Poisson distribution. Before the compact septal clusters became clearly distinguishable (after 3 generations) they were not excluded from the grain distribution analysed (Table 1) which diminishes the probability of the distribution being Poisson at 1.5 generations. After five generations the probability of finding a Poisson distribution was less than 0.05%, confirming the non-random distribution seen in the autoradiographs.

Immediately after the chase of pulse labelled cultures of the parent strain and Ni15 (Fig. 2) grain distribution was not distinguishable from Poisson (Table 1), in agreement with previous conclusions⁹. However, 1.5 generations after chasing, grain distributions for both strains had become non-Poisson, demonstrating segregation of the pulse-incorporated material from unlabelled wall. This strongly supports a localised pattern of insertion of new wall material, common to both strains. Because more than 90% of the pulse label was still present in parent cell wall 1.5 generations after the chase¹¹, a selective localised degradation of label due to turnover cannot account for the observed segregation. After 3 generations the grain distribution in Ni15 remained clearly non-Poisson.

Although these results confirm the pattern obtained with continuously labelled cells, it is clear that segregation of the pulse label occurs much earlier. Possibly segregation is masked for up to 3 generations after chasing a continuously labelled culture. This agrees with the model¹¹ in which old wall 'spreads', forming a progressively thinner layer occupying an increasing area at the outer surface of the wall. Spreading cannot continue indefinitely because segregation is evident within five generations. The rapid segregation of a pulse label suggests that wall material of the same age, inserted locally, undergoes more limited 'spreading'. The presence of more than

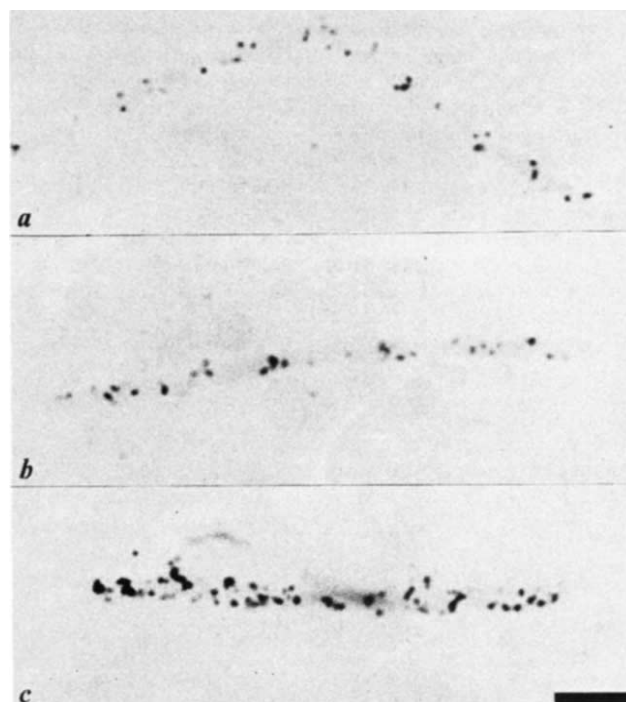


Fig. 2 Chase of pulse labelled cells. Exponentially growing cultures of 168 *trp*⁺ and Ni15 were filtered at an A_{540} of 0.06 and resuspended in warmed medium containing ^3H -GlcNAc (specific activity 3 Ci mmol⁻¹). After 1 min cells were re-filtered, washed and resuspended in unlabelled medium. Samples withdrawn 0, 1.5 and 3 generations later were treated and analysed as described in the legend to Fig. 1. *a*, Ni15 at 1.5 generations; *b*, 168 at 1.5 generations; *c*, 168 at 0 generation. The bar is equivalent to 5 μm .

one site of wall growth per cell, in cells growing in rich medium, could also delay the appearance of half-cells devoid of grains during the chase of continuously labelled cultures.

Our results do not contradict recent findings in failing to show segregation during the initial period of chasing. However,

Table 1 Statistical analysis of grain distributions after chasing ^3H -GlcNAc-labelled cultures of *B. subtilis* 168 and mutant Ni15

Time after chasing in generations (min)	Ni15								168							
	Continuous labelling*								Pulse labelling†							
	Class 1 <i>m</i>	Class 2 <i>A</i> (%)	<i>m</i>	<i>A</i> (%)	<i>M</i>	<i>R</i>	<i>m</i>	<i>A</i> (%)	Class 1 <i>m</i>	Class 2 <i>A</i> (%)	<i>M</i>	<i>R</i>	<i>m</i>	<i>A</i> (%)	<i>m</i>	<i>A</i> (%)
0(0)	5.4	85	7.5	98	6.2	1	6.8	52	10.0	60	8.6	1	4.8	40	6.6	52
1.5 (30)	4.4	80	6.7	33	5.4	1.0	2.7	0.05	3.5	7	3.1	1.1	2.2	<0.05	3.0	0.4
3 (60)	3.8	42	5.5	46	4.8	1.0	1.0	0.05	1.3	3	1.1	1.2				
	3.8‡	46	6.3	4	5.1	—										
5 (100)	1.8	<0.05	2.7	<0.05	2.5	0.8										
	1.7‡	<0.05	2.5	<0.05	2.2	—										

The grain distributions per half-cell obtained as described in Figs 1 and 2 were compared with that of Poisson by the χ^2 test. To diminish the variation in grains per half-cell due solely to variation in cell length, the population was divided into two classes, following previous practice⁹: cells shorter than (class 1), and cells longer than or equal to (class 2) the mean length (4.2 μm). Of 1,600 cells measured the shortest was 2.0 μm and the longest, 8.0 μm . *m*, Mean number of grains per half-cell for each class (obtained on a population of between 80 and 360 half-cells); *A*, probability that the distribution is Poisson; *M*, mean number of grains per half-cell for the whole population. Dense clusters (Fig. 1e) associated with septal regions were excluded from the distributions at three and five generations (see text). At five generations the frequency of septal clusters (one per 15 cells) and the proportion of grains (13%) in these clusters were determined on 1,330 cells (four experiments). *R*, ratio of *M*, found at each time, to that calculated from the value at 0 generation, the specific activity used, an assumed dilution of 0.5 per generation and the extent of wall turnover obtained from published results¹¹.

*Total loss of radioactivity, due to turnover, is about 20% after 5 generations¹¹.

†Loss of radioactivity due to turnover from Ni15 after 3 generations, and from 168 after 1.5 generations is about 15% and 5% respectively¹¹.

‡Separate experiments.

by extending observation over five generations, we have demonstrated a clear segregation of old and new wall. We conclude that the insertion of new cell wall material in *B. subtilis* is localised and occurs at a few sites per cell. We are investigating the relationship between the segregation pattern of cell wall and that of the bacterial chromosome.

This work was supported by a grant to D.K. and V. Bonifas from the Swiss National Science Foundation (no. 3.417.74) and by the Roche Research Foundation for Scientific Exchange and Biomedical collaboration with Switzerland. We thank Professor V. Bonifas for facilities. Part of this work has been reported already¹³.

H. M. POOLEY
J.-M. SCHLAEPPI
D. KARAMATA

*Institut de Microbiologie,
Université de Lausanne,
19 Rue Cesar-Roux,
1005 Lausanne, Switzerland*

Received 13 February; accepted 30 May 1978.

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Lack of transformation of murine thymocytes by thymic epithelium

THE normal thymus consists of two basic components: a reticular-epithelial component derived from the third and fourth bronchial pouches¹ and a lymphoid component from stem cells which migrate into the embryonal thymus from fetal liver² and later from bone marrow³. Two types of thymic reticular cells have been observed by electron microscopy: epithelial reticular cells and mesenchymal reticular cells or macrophages. The villous epithelial cells of the cortex and the cystic epithelial cells of the medulla were shown to be important for T cell differentiation⁴. Cultures of thymus epithelial cells were shown to induce the differentiation of precursor cells into certain mature T lymphocytes^{5,6}. Recent studies have suggested that thymic epithelium cells (derived from leukaemic thymus) were capable of providing the leukaemogenic signal and thereby inducing *in vitro* leukaemia transformation of normal thymocytes. Thus, it was claimed that young AKR thymocytes cultured on thymus epithelium monolayers from aged AKR mice could induce leukaemia when injected into normal young recipients⁷. Similarly, monolayers of thymus epithelium cells, derived from radiation leukaemia virus-induced thymomas in C57BL/6 mice could bring about transformation of normal C57BL/6 thymocytes, thereby demonstrating *in vitro* leukaemogenesis⁸. Our aim here was to study further the possible role of the thymic epithelial reticulum (TER) in leukaemogenesis and we present evidence that the leukaemic cells are not derived from the thymus lymphocytes, nor from a 'transformation capacity' of the epithelial cells themselves, but that they are already present within phagocytic TER cells. We used the thymic epithelium monolayer-thymocyte co-cultivation method^{7,8} and the transplantation bioassay genotype analysis method⁹ (used to demonstrate the origin of the developing leukaemias following injection of thymocytes or thymic epithelium cells into appropriate recipients).

TER monolayers were prepared from thymomas induced in 6–8-week-old C57BL/6 or (BALB/c×C57BL/6)F₁ mice by

the radiation leukaemia virus variants (RadLV) or fractionated radiation^{10,11}, or from nonleukaemic thymuses of 10–12-week-old C57BL/6 mice previously (30 d) injected intrathymically with RadLV. Thymus epithelial reticulum monolayers were grown as previously described¹². Thymomas, virus-inoculated or normal thymuses were removed aseptically into chilled phosphate-buffered saline, cut into small pieces and trypsinised for 20 min with 0.25% trypsin. The trypsinised cells were washed twice with Waymouth culture medium supplemented with 5% FCS. Thereafter, the cells were resuspended in Waymouth medium 5% FCS and plated in 60-mm Falcon Petri dishes. After 48 h half of the Waymouth medium was removed and Dulbecco's modified Eagle's medium containing 10% fetal calf serum added. The epithelial reticulum cells were attached to the Petri dish and formed a complete monolayer within 10 d. Several weeks after establishing the monolayer cultures (when leukaemic cells were no longer seen) thymocytes (20–50×10⁶ taken from 6–8-week-old C57BL/6 or (BALB/c×C57BL/6)F₁ mice) were overlaid on the appropriate TER monolayer for 72 h. Most of the TER cells were retained on the plate, but some cells were detached and mixed with the thymocytes. The free thymocytes were removed by gentle shaking of the plates. No matter how gently the thymocytes were removed they always included some large phagocytic cells derived from the monolayer. 1–2×10⁶ culture-viable thymocytes were then injected intraperitoneally into C57BL/6 or (BALB/c×C57BL/6)F₁, 6–8-week old recipients previously exposed to 400 R whole body irradiation.

The use of hybrid mice as cell recipients enabled us to identify the genotype of the developing leukaemia, whether of thymocyte, epithelial reticulum or recipient origin (by transplanting the ensuing tumours developing in F₁ hybrid mice into F₁ and parental strains). Leukaemias developing following injection of thymocytes overlaid on monolayers were always of the monolayer origin. Thymocytes from (BALB/c×C57BL/6)F₁ mice overlaid on a C57BL/6 monolayer (Table 1, group 4) caused leukaemia development in the F₁ recipients of C57BL/6 monolayer origin, whereas C57BL/6 thymocytes overlaid on F₁-derived monolayers induced leukaemias of F₁ monolayer origin (Table 1, group 7). Thymic epithelial cells derived from thymomas induced in C57BL/6 mice by RadLV (Table 1, groups 1, 2) or in F₁ mice by RadLV plus 400 R (Table 1, groups 5, 6) when injected into C57BL/6 or F₁ recipients induced a high incidence of leukaemia within 15–24 d, the leukaemias being always of the same origin as the epithelial monolayer cells. Thymus epithelial reticulum cells were shown to be resistant to radiation (up to 5,000 R), in contrast to the radiosensitivity of thymocytes¹³. Exposure of TER monolayers to 2,000 R abrogated leukaemia induction by thymocytes overlaid on them, whereas a high incidence leukaemia was observed in the matching control group (Table 1, groups 9, 10 compared with control group 7).

Fig. 1 Immunofluorescence micrograph of alcohol-fixed thymus epithelial reticulum of RadLV injected nonleukaemic thymus. The reticulum cells are gp71-positive. Magnification×400.

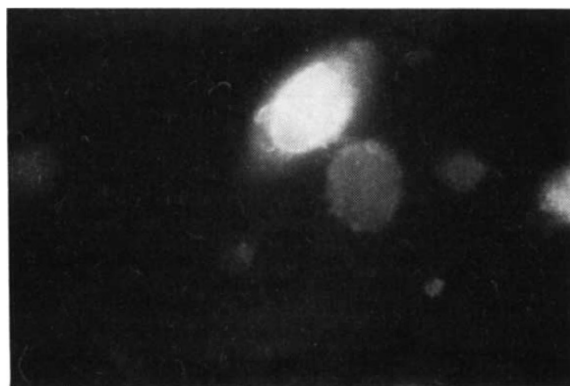


Table 1 Leukaemogenic potential of thymocytes cultured on thymus epithelium monolayers

Group no.	Strain	Origin of TER* Host treatment	Thymocyte origin	Recipients* of TER or thymocytes	Leukaemia incidence in recipients	Genotype analysis of leukaemias†		Leukaemia origin
						B1/6	F1	
1	B1/6	RadLV-leukaemic	—	B1/6	4/4 (100%) (24)†	—	—	B1/6
2	B1/6	RadLV-leukaemic	—	F ₁	9/9 (100%) (15)	9/9	0/9	B1/6
3	B1/6	RadLV-leukaemic	B1/6	B1/6	55/ (100%) (37)	—	—	B1/6
4	B1/6	RadLV-leukaemic	F ₁	F ₁	2/3 (66%) (37)	2/2	0/2	B1/6
5	F ₁	RadLV + 400R-leukaemic	—	F ₁	2/2 (100%) (24)	—	—	F ₁
6	F ₁	RadLV + 400R-leukaemic	—	B1/6	0/5 (0%)	—	—	F ₁
7	F ₁	RadLV + 400R-leukaemic	B1/6	F ₁	2/3 (66%) (24)	0/2	2/2	F ₁
8	F ₁	RadLV + 400R-leukaemic	B1/6	B1/6	0/6 (0%)	—	—	F ₁
9	F ₁	RadLV + 400R-leukaemic + 2,000 R	B1/6	F ₁	0/6 (0%)	—	—	F ₁
10	F ₁	RadLV + 400R-leukaemic + 2,000 R	B1/6	B1/6	0/6 (0%)	—	—	F ₁
11	B1/6	RadLV-preleukaemic	—	B1/6	2/4 (50%) (90)	—	—	B1/6
12	B1/6	RadLV-preleukaemic	—	F ₁	3/8 (37%) (105)	0/3	3/3	F ₁
13	B1/6	RadLV-preleukaemic	B1/6	B1/6	4/4 (100%) (90)	—	—	B1/6
14	B1/6	RadLV-preleukaemic	B1/6	F ₁	2/3 (66%) (60)	0/2	2/2	F ₁
15	B1/6	RadLV-preleukaemic	F ₁	B1/6	5/5 (100%) (85)	3/3	0/3	B1/6
16	B1/6	RadLV-preleukaemic	F ₁	F ₁	3/6 (50%) (105)	0/2	2/2	F ₁
17	B1/6	170 × 4 leukaemic	F ₁	F ₁	8/8 (100%) (55)	5/5	0/5	B1/6

TER, thymus epithelial reticulum; B1/6 = C57BL/6; F₁ = (BALB/c × C57BL/6)F₁. Leukaemic or RadLV-injected TER were grown in cultures as described elsewhere¹². The cultures were kept *in vitro* for 25–30 d.

* Normal thymocytes from the appropriate strains (6–8 weeks old) were incubated on TER for 72 h. The overlaid cells were then collected and 1–2 × 10⁶ cells were injected intraperitoneally into the appropriate recipients previously exposed to 400 R whole body irradiation. In some experiments the TER cells alone were injected intraperitoneally.

† Average latent period in days.

‡ The genotype analysis of the leukaemia occurring in the recipient was done by transplanting the leukaemic cells into the parental and F₁ hybrid strains.

It has been shown¹⁴ that within 30 d of intrathymic RadLV administration preleukaemic-leukaemic cells have not yet been established in the thymus, although RadLV replication is maintained in these thymocytes^{15,16}. Thymus epithelial reticular monolayers established from thymuses removed 30 d after RadLV intrathymic inoculation were found to maintain virus replication in the following test: alcohol-fixed monolayer was subjected to rabbit anti-AKR gp71 and thereafter to fluorescein isothiocyanate (FITC)-goat anti-rabbit IgG. Figure 1 shows that these TER cells had bright fluorescent cytoplasm, indicating intracellular virus associated antigen. We therefore tested whether TER monolayers derived from such nonleukaemic RadLV-containing thymuses would contribute to thymocyte transformation. As seen in Table 1, groups 11–16, the developing leukaemias following thymocyte or TER cell trans-

fer were always of recipient origin. Thus, thymocytes and epithelial cells served as a vector for RadLV transfer into the recipient mice. The long latent period for leukaemia development observed in groups 11–16 (Table 1) reflected *de novo* leukaemia induction by RadLV as opposed to the short latent period following transfer of leukaemic cells (Table 1, groups 1–7). Cell-free extracts from C57BL/6 radiation-induced thymomas were found to have only weakly leukaemogenic activity¹⁷ and to lack the expression of endogenous ecotropic MuLV (ref. 18). It therefore seemed of interest to test whether TER derived from such thymomas could transform thymocytes. As can be seen in Table 1, group 17, thymocytes from (BALB/c × C57BL/6)F₁ overlaid on the C57BL/6 monolayer caused leukaemia development in 100% of the F₁ recipients, all leukaemias being of C57BL/6 monolayer origin.

Table 2 Analysis of leukaemias developing following injection of thymocytes cultured on leukaemic AKR TER monolayers

Group no.	Origin of TER	Thymocyte origin	Recipients of TER* or thymocytes	Leukaemia incidence in recipients	Thy 1 analysis on leukaemic cells† (index in %)	
					Thy 1.1	Thy 1.2
1	Leuk. AKR/J (Thy 1.1)	AKR/Cu (Thy 1.2)	AKR/Cu (Thy 1.2)	4/4 (100%) (34)†	65–77	—
2	Leuk. AKR/Cu (Thy 1.2)	AKR/J (Thy 1.1)	AKR/J (Thy 1.1)	3/3 (100%) (39)	—	80–85
3	Leuk. AKR/Cu (Thy 1.2)	AKR/J (Thy 1.1)	AKR/Cu (Thy 1.2)	4/4 (100%) (34)	—	85–90
4	Leuk. AKR/J (Thy 1.1)	—	AKR/Cu (Thy 1.2)	5/6 (80%) (21)	80–95	—
5	Leuk. AKR/J (Thy 1.1)	—	AKR/J (Thy 1.1)	2/2 (100%) (27)	95	—
6	Leuk. AKR/Cu (Thy 1.2)	—	AKR/J (Thy 1.1)	3/4 (75%) (31)	—	80

Leukaemic (leuk.) TER from spontaneous leukaemias of AKR/Cu or AKR/J mice were grown in cultures as described. The cultures were kept 25–30 d.

* Normal thymocytes from appropriate strains (6–8-weeks-old) were incubated on TER for 72 h. The overlaid cells were then collected and 1–2 × 10⁶ cells were injected intraperitoneally into appropriate recipients previously exposed to 400 R whole body irradiation. In some experiments TER cells alone were injected.

† Average latent period in days.

‡ Thy 1 analysis was carried out on individual leukaemias with anti-Thy 1.2 antiserum titre of 1:64 (AKR/J anti-C3H thymocytes antiserum) and anti-Thy 1.1 (Searle) with a titre of 1:8. 3 × 10⁵ cells in 50 µl were mixed with 50 µl of antiserum incubated for 30 min at 4°C. The tubes were centrifuged, supernatant was discarded and 50 µl of guinea pig complement was added. The tubes were incubated at 37°C for 30 min. Viable cells were determined by Trypan blue exclusion. The results are reported as the cytotoxic index. Cytotoxic index = (dead cell (experimental) – dead cell (control))/viable cells (control).

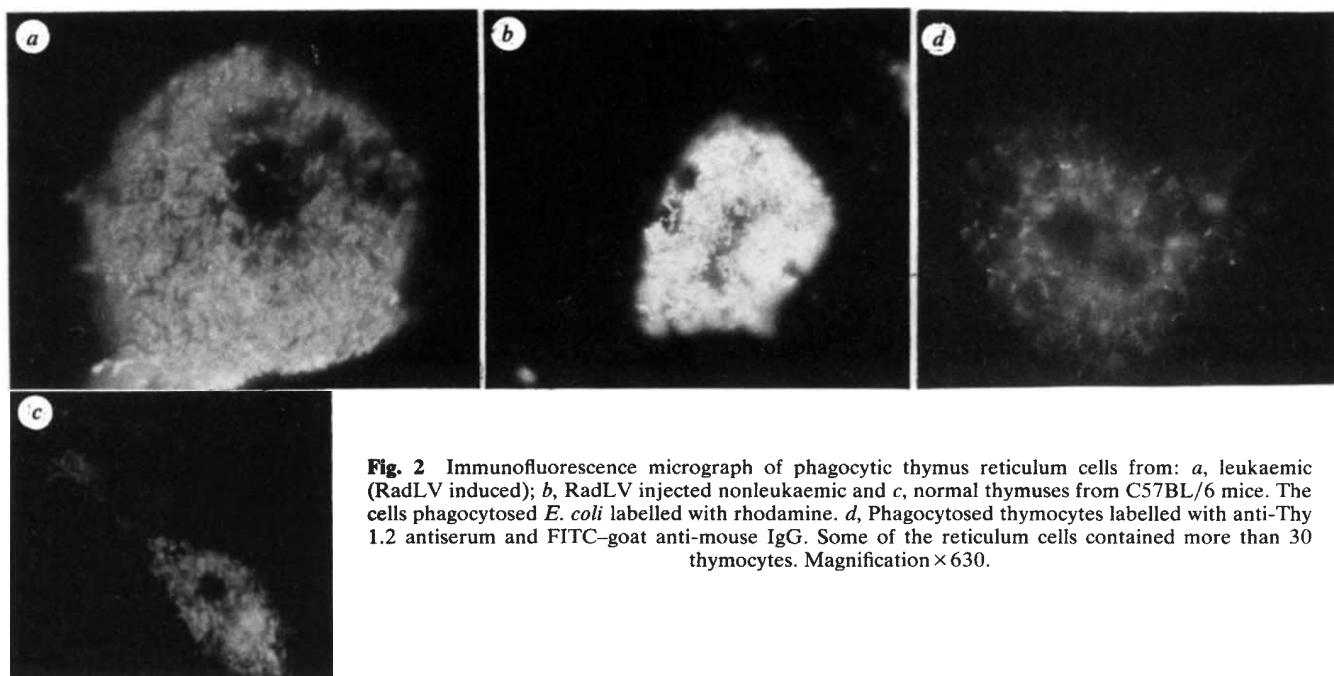


Fig. 2 Immunofluorescence micrograph of phagocytic thymus reticulum cells from: *a*, leukaemic (RadLV induced); *b*, RadLV injected nonleukaemic and *c*, normal thymuses from C57BL/6 mice. The cells phagocytosed *E. coli* labelled with rhodamine. *d*, Phagocytosed thymocytes labelled with anti-Thy 1.2 antiserum and FITC-goat anti-mouse IgG. Some of the reticulum cells contained more than 30 thymocytes. Magnification $\times 630$.

All our results indicated unequivocally that leukaemic cells derived from thymic epithelium monolayers originally obtained from thymomas were transferred with the overlaid normal thymocytes. These epithelium-derived leukaemic cells 'hidden' in the phagocytic cells rather than *in vitro* transformed thymocytes were responsible for leukaemia development in recipient mice. The crucial debatable point on the basis of the present results and those obtained in previous studies by Waksal *et al.*⁷ and Haas *et al.*⁸ relates to the origin of the developing leukaemias. In those studies^{7,8} no experimental data were provided concerning the leukaemogenic potential of TER cells alone, although they suggested that TER cells provided the leukaemogenic signal and thereby induced *in vitro* leukaemia transformation. In the studies of Haas *et al.*⁸ the proof that leukaemia-inducing cells were not actually derived from cells hidden among the TER monolayers was provided by showing that C3H thymocytes cultured on C57BL/6 monolayers had no leukaemogenic potential. These results could be attributed to allogeneic cell killing in a C3H-C57BL/6 combination. In studies by Waksal *et al.*⁷ analysis of the origin of leukaemias induced by infecting thymocytes of AKR/Cu or AKR/J overlaid on TER derived from old AKR/J or AKR/Cu (using the different θ antigen markers, Thy 1.1 or Thy 1.2 on these thymocytes to define the leukaemia origin) indicated the thymocyte tumour origin. The results of similar analyses which we carried out (summarised in Table 2) indicated that the leukaemias developing following injection of TER cells alone or thymocytes overlaid on TER monolayers were of thymic epithelium origin.

Our results suggest that thymic epithelial reticulum might be considered as a favourable site for the proliferation of transformed cells. Indeed, feeder layers containing reticuloendothelial cells were found to be required for the growth of primary explants of lymphomas¹⁹. These results actually agree with other reports concerned with the presence of phagocytic cells among thymic reticular cells observed *in vivo* or *in vitro*. Clark²⁰ observed large macrophage-like phagocytic cells in the thymus. Metcalf *et al.*²¹ observed large phagocytic cells, derived from normal reticuloendothelial cells, in thymus, lymph nodes and spleen of leukaemic AKR mice, but not in normal or preleukaemic AKR thymus. Electron microscopic studies on such phagocytic cells have shown them to contain large numbers of leukaemia virus particles²². Izard and de Harven²³ have described dense reticular cells in the thymus and

lymph nodes of leukaemic AKR mice (rare in normal AKR mice), having long cytoplasmic processes which often completely surrounded many thymocytes and lymphocytes. Similarly, Ioachim and Furth²⁴ showed the presence of viable lymphoblasts undergoing mitosis within the cytoplasm of reticulum cells maintained in tissue cultures (derived from thymic lymphomas induced in rats by the Gross passage A virus). Further evidence concerning the competence of normal and leukaemic thymus epithelial reticulum monolayers to phagocytose different substances was provided in the following experiments. Thymic epithelium monolayers derived from leukaemic, virus-containing nonleukaemic or normal thymuses were fed with carbonyl iron, *Escherichia coli* labelled with rhodamine, normal or leukaemic thymocytes exposed to anti-Thy 1.2 antisera, and thereafter stained with FITC-goat anti-mouse IgG. Phagocytosis (Fig. 2) was seen in all the different monolayers, although the number of phagocytic cells in the monolayers derived from thymomas was 10 times more than that in monolayers derived from normal or virus-containing thymuses. Many large cells of the monolayer were filled with labelled thymocytes, sometimes with more than 30 cells, looking like clusters of grapes (Fig. 2*d*).

In conclusion, the *in vitro* 'transformation capacity' of thymic epithelium cells, suggested by some investigations^{7,8}, has not been confirmed in the present studies. The phagocytic cells present within the epithelial reticulum contain leukaemic cells. The presence of thymotropic, ecotropic and xenotropic viruses in thymic epithelial monolayers derived from RadLV-induced thymomas⁸ could be due to RadLV-induced leukaemic cells phagocytosed and hidden among the cells establishing the monolayer.

We thank Rita Krautgamer for her technical assistance. This work was supported by MSPHS research contract no. 1-CB-43930. Rabbit anti-AKR gp71 was provided by Dr J. N. Ihle and *E. coli* labelled with rhodamine by Dr B. Geiger.

ALPHA PELED
NECHAMA HARAN-GHERA

Department of Chemical Immunology,
Weizmann Institute of Science,
Rehovot, Israel

Received 20 February; accepted 30 May 1978.

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Generation of cytotoxic T lymphocytes to autologous human leukaemia cells by sensitisation to pooled allogeneic normal cells

MANY murine and human tumour cells express cell surface antigens cross-reactive with histocompatibility antigens that are expressed on normal cells of unrelated individuals of the species^{1–7}. One approach for generating cytotoxic lymphocytes capable of lysing autologous or syngeneic tumour cells there-

fore might be to sensitise to the appropriate cross-reactive alloantigen(s). Results obtained in our laboratory indicate that lymphocytes sensitised in mixed leukocyte culture to a pool of normal cells from 20 unrelated individuals⁸ lyse virtually all allogeneic target cells⁹, suggesting that the 'pool' presents essentially all the target determinants for cytotoxic T lymphocytes (CTL) expressed on normal human cells. We therefore hypothesised that sensitisation of T cells to the 'pool' may be a means for generating CTL capable of lysing autologous morphologically transformed cells without the need to experimentally define which alloantigen specificities are expressed on particular transformed cells¹⁰. One model in which we have recently demonstrated the efficacy of pool sensitisation involves Epstein-Barr virus transformed human lymphoblastoid cell lines (LCL); normal T lymphocytes sensitised *in vitro* against the pool lyse autologous ⁵¹Cr-labelled LCL cells but not autologous normal T cells, B cells or mitogen-induced blast cells¹¹. Studies were thus undertaken to determine whether leukaemia cells, which are incapable of stimulating cytotoxic responses in autologous lymphocytes^{12–14}, would be lysed by T cells from patients sensitised *in vitro* with pooled allogeneic cells. We report here that 'pool' sensitisation of T cells from patients with hairy cell leukaemia (leukaemic reticuloendotheliosis) gives rise to CTL that lyse autologous leukaemia cells but not autologous normal lymphocytes.

Hairy cell leukaemia cells were obtained from the spleens of two adult male patients (designated P1 and P2) at the time of therapeutic splenectomy. Spleen cells were layered on Ficoll-Hypaque gradients, and cells at the interface of the gradients (consisting of >80% leukaemic cells as determined by morphology and tartrate-resistant acid phosphatase staining¹⁵) were frozen in RPMI-1640 medium containing 25 mM

Table 1 Lysis of autologous leukaemia cells by T cells sensitised to pooled allogeneic normal cells

Responding T cells	Stimulating cells	% ⁵¹ Cr release ± s.d. from target cells			
		P1's splenic leukaemic cells	P1's peripheral leukaemic cells	P1's peripheral T cells	A's peripheral T cells
Expt a P1	P1-L _x (P1's leukaemia cells)	1.7 ± 2.3		0.5 ± 3.0	
	Pool _x	24.5 ± 1.8		0.8 ± 2.5	
Expt b P1	P1-L _x (P1's leukaemic cells)	–1.0 ± 0.5			
	A _x	5.3 ± 2.6			37.7 ± 3.9
	Pool _x	19.0 ± 3.7	21.1 ± 0.8	–1.5 ± 2.7	36.4 ± 5.6
	A	32.8 ± 2.6			
	P1-L _x	29.5 ± 2.8	45.5 ± 3.1	15.8 ± 2.7	–9.2 ± 3.4
	Pool _x				
Expt c P2		P2's splenic leukaemic cells	P2's peripheral leukaemic cells	P2's peripheral T cells	A's peripheral T cells
	P2-L _x (P2's leukaemic cells)	–2.6 ± 1.7			
	Pool _x	18.1 ± 5.6	27.2 ± 3.6		
	A	25.2 ± 4.1	21.0 ± 2.6	16.8 ± 4.9	–3.6 ± 2.0
Expt d P2	Pool _x	18.9 ± 1.5		–0.3 ± 2.5	
	B	32.7 ± 2.9		41.0 ± 5.4	

T cells from peripheral blood of the normal individuals (A and B) and the hairy cell leukaemia patients (P1 and P2) were isolated as described in the text. The published micromethod for generating human CTL (ref. 17) was followed. Briefly, 1×10^5 responding T cells were cultured in several replicate microwells with either 1×10^5 X-irradiated (2,500R) allogeneic cells from a single individual (A_x), with 1×10^5 X-irradiated (4,000R) splenic leukaemic cells (L_x) or with 1×10^5 X-irradiated (2,500R) allogeneic normal cells pooled from 20 individuals (that is, 5×10^3 cells from each of 20 unrelated individuals). On day 7, cell-mediated cytotoxicity was measured by the ⁵¹Cr release assay¹⁷ using, as target cells, T cells or peripheral leukaemic cells that were cultured for 7 d, or splenic leukaemic cells that were thawed one day before testing. The target cells were labelled with ⁵¹Cr, and 1×10^6 labelled target cells were added to each of four replicate microwells containing the sensitised cells¹⁷, resulting in effector to target cell ratios of 10–20:1. The ⁵¹Cr release assays were terminated after 7 h and the percentage ⁵¹Cr released from target cells was calculated as described previously¹⁷. T cells, from the normal individuals or patients, that were cultured in the absence of stimulating cells were not cytotoxic for leukaemic cells (–2.3 ± 1.4 to 0.6 ± 2.0% ⁵¹Cr release). T cells (1×10^5 cells per well) from the patients were stimulated with four concentrations of autologous X-irradiated (4,000R) leukaemic cells (ranging from 0.25×10^5 to 2×10^5 cells per well), and in no case was cytotoxicity against autologous leukaemic cells detected.

HEPES, 25% heat-inactivated normal human serum and 10% dimethyl sulphoxide. Heparinised peripheral blood was obtained from these two patients, who received no therapy, 1–12 weeks after splenectomy. Mononuclear cells from the patients and normal individuals were separated from the blood by Ficoll–Hypaque sedimentation. T lymphocytes were isolated from the normal individuals' mononuclear cells by rosetting with sheep red blood cells (SRBC) as described previously¹⁶. The same methods were used to obtain T cells and non-T cells (consisting of non-SRBC-rosetting peripheral leukaemia cells) from the patients' blood. T cells from the normal individuals and the patients were stimulated in mixed leukocyte culture with either X-irradiated leukaemia cells, allogeneic cells derived from a single individual, or allogeneic cells prepared by pooling equal numbers of lymphocytes from 20 normal unrelated individuals⁸. The sensitised cells were subsequently tested for their ability to lyse the leukaemia cells and normal cells by the cell-mediated ⁵¹Cr release assay as described in Table 1.

The results of the first two experiments shown in Table 1 demonstrate that sensitisation of T cells from patient 1 (P1) with the pooled allogeneic cells resulted in the generation of cytotoxicity against autologous splenic and circulating leukaemic cells but not against autologous normal T cells. Sensitisation of the T cells of patient 1 with allogeneic cells derived from a single individual (A_x) resulted in minimal cytotoxicity against the autologous leukaemia cells compared with that mediated by pool-sensitised cells, whereas cytotoxicity against A's target cells by lymphocytes sensitised against A_x cells was equivalent to that mediated by the pool-sensitised cells. The minimal ability of lymphocytes from a single allogeneic individual to stimulate T cells of patients to develop into CTL capable of killing autologous leukaemia cells is in agreement with previous findings^{12–14} and with our results in the LCL model system, which indicate that lymphocytes stimulated with single allogeneic cells are rarely cytotoxic for autologous LCL cells¹¹. Furthermore, stimulation of the T cells of patient 1 with X-

irradiated autologous leukaemic cells failed to result in the generation of cytotoxicity against the autologous leukaemia cells.

The results of experiments c and d shown in Table 1 likewise demonstrate, in the other patient with hairy cell leukaemia (P2), that T cells sensitised with the pool were cytotoxic for both autologous splenic and circulating leukaemic cells but were not cytotoxic for normal autologous T cells. It should be noted, however, that lymphocytes from normal individuals, after sensitisation with the pool, were cytotoxic for the patients' T cells as well as the leukaemic cells indicating that T cells are sensitive to cell-mediated lysis. The failure of both patients' leukaemia cells to stimulate cytotoxic responses in autologous normal lymphocytes, despite their ability to stimulate cytotoxic responses in allogeneic lymphocytes, agrees with our findings¹² (Zarling *et al.*, unpublished observations) and those of others^{13,14} in over 80 patients with a variety of leukaemias.

Consistent with the finding that pool-sensitised lymphocytes are cytotoxic for autologous leukaemia cells but not for normal autologous lymphocytes, are the results shown in Table 2 indicating that unlabelled autologous leukaemic cells, but not autologous normal T cells, competitively inhibit lysis of ⁵¹Cr-labelled autologous leukaemia cells mediated by pool-sensitised cells. Normal unlabelled lymphocytes are, however, effective in blocking lysis of ⁵¹Cr-labelled target cells mediated by CTL sensitised against relative alloantigens^{9,11}. That the leukaemic cells from P1 do not non-specifically block cell-mediated lysis is demonstrated at the bottom of Table 2; T cells and leukaemic cells from P1 inhibited equally, and only to a small extent (19–20%), lysis of ⁵¹Cr-labelled leukaemia cells from P2 mediated by pool-sensitised T cells from individual B. The results of the cytotoxicity and blocking experiments indicate therefore that pool-sensitised T cells are directed against target determinants detected on autologous leukaemic cells, but not on autologous normal lymphocytes that cross-react with target determinants present in the pool.

Several explanations have been put forward to account for the findings that leukaemia cells, which express cytotoxic target determinants, do not, in themselves, induce cytotoxic responses in autologous lymphocytes^{12–14}. Pool sensitisation, utilising the cross-reactivity between the normal alloantigens of the pool and target antigens on the leukaemia cells, may bypass the difficulties attendant on attempts to generate CTL by utilising leukaemia cells as the sensitising cells. Results of further experiments will determine whether sensitisation of T cells with pooled allogeneic normal cells may be a useful method for generating specific cytotoxic lymphocytes directed against autologous malignant cells of other leukaemias or tumours.

We thank L. Eskra, R. Klopp, V. Baker, C. Arndt and K. Heim for assistance, and B. Hasty for providing patients' specimens. This work was supported in part by NIH grants CA-20409, CA-14520, CA-16836 and AI-11576.

JOYCE M. ZARLING
H. IAN ROBINS
PETER C. RAICH
FRITZ H. BACH
MARILYN L. BACH

*Immunobiology Research Center and
Departments of Human Oncology, Medicine,
Surgery, Medical Genetics and Pediatrics,
University of Wisconsin,
Madison, Wisconsin 53706*

Received 1 May; accepted 15 May 1978.

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Table 2 Ability of unlabelled leukaemic and normal cells to block lysis of ⁵¹Cr-labelled autologous leukaemic cells by T cells sensitised to the pool

Responding T cells	Stimulating cells	Unlabelled blocking cells	% ⁵¹ Cr release ± s.d.	% Inhibition of lysis
			P1 leukaemic cells	
P1	Pool _x	None	16.8 ± 3.8	—
		P1 splenic leukaemic cells	2.4 ± 1.3	86
		P1 peripheral leukaemic cells	0.8 ± 0.9	95
		P1 peripheral T cells	17.3 ± 3.8	0
			P2 leukaemic cells	
B	Pool _x	None	32.7 ± 2.9	—
		P1 splenic leukaemic cells	26.5 ± 3.0	19
		P1 peripheral T cells	27.3 ± 1.6	20

T cells from P1 and normal individual B were sensitised to the pooled allogeneic cells (pool_x) as described in Table 1. On day 7, the effector cells were tested for their ability to lyse ⁵¹Cr-labelled splenic leukaemic cells from P1 or P2 at a ratio of effector: target cells of 20:1 in the presence or absence of 20 unlabelled 'blocking cells' per ⁵¹Cr-labelled target cell. The ⁵¹Cr release assay was terminated after 7 h. The % inhibition of lysis was determined by comparing the % cell-mediated ⁵¹Cr released from target cells in the presence or absence of unlabelled blocking cells.

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Opposing effects of tumour promoters on erythroid differentiation

TUMOUR promoters are compounds which are not carcinogens but which can induce tumours in mice treated with a subcarcinogenic dose of a chemical carcinogen^{1,2}. The mechanism of action of tumour promoters *in vivo* is unknown, although recent reports suggest that they may inhibit terminal differentiation of certain cells *in vitro*. Myogenesis of chick embryo myoblasts³, spontaneous and induced erythroid differentiation of Friend virus-transformed proerythroid cells^{4,5} and lipogenesis in a clone of BALB/c 3T3 preadipocyte cells are examples of *in vitro* differentiation processes inhibited by tumour promoters. Unfortunately, inhibition of differentiation by tumour promoters has not yet been shown to occur *in vivo*. It is also uncertain whether the cells used in the above studies are representative of the actual target cells for tumour promoters *in vivo*. We report here that tumour promoters can induce as well as inhibit differentiation of virus-transformed proerythroid cells *in vitro*. Detection of the opposing effects depends on measuring tumour promoter action in more than one virus-transformed proerythroid cell line.

We studied the effect of the potent tumour promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and its inactive parent compound, phorbol, on erythroid differentiation of virus-transformed proerythroid cells. Three cell lines were used: Friend virus-transformed proerythroid line^{7,8}, CI 745, and two Rauscher virus-transformed proerythroid lines^{9,11}, RV-133 and RV-187. All three can be induced to differentiate along the erythroid pathway by exposure to compounds (inducers) such as dimethylsulphoxide (DMSO)^{10–12}, hypoxanthine¹², and acetamide¹².

When CI 745 cells were grown in the presence of 1% DMSO, 0.1% hypoxanthine or 1% acetamide, approximately 80% of the cells became haemoglobinised as determined by the benzidine test^{10,12}. The simultaneous addition of TPA, however, inhibited induction of differentiation (Table 1). TPA also inhibited spontaneous differentiation of CI 745 cells. These results agree with other reports^{4,5}, although the levels of inhibition were less in our studies. This may be due to differences among the cell lines maintained in the different laboratories, although the Friend virus-transformed proerythroid cells in all three studies originated from the parent line Gm-86 clone 745. Phorbol, the nonpromoter parent

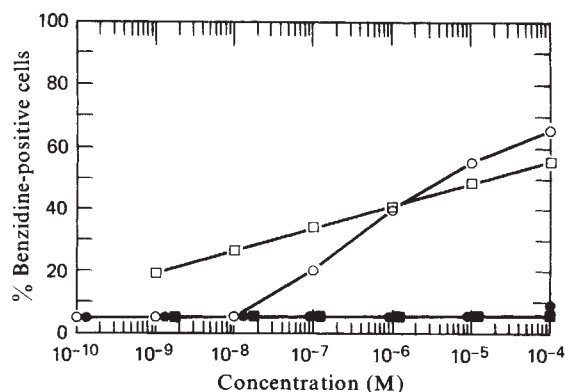


Fig. 1 Induction of differentiation of Rauscher virus-transformed proerythroid cells by TPA. Induction of differentiation was assayed as previously described¹². In brief, cells were seeded in microtitre plates at a concentration of 2×10^2 cells per 0.1 ml per well. After 24 h of incubation, 0.1 ml of the appropriate concentration of TPA or phorbol diluted in culture medium was added. Both compounds had been kept at 4°C in the dark until used. After 5 days of incubation cultures were assayed for haemoglobin synthesis by the benzidine test. Photographs were taken and the number of benzidine-positive cells was determined out of about 200–500 cells counted. Symbols were: TPA, open figure and phorbol, closed figure; RV-133, circles and RV-187, squares.

compound, had no effect on either spontaneous or induced differentiation. Our data confirm that 10^{-7} – 10^{-9} M TPA inhibited differentiation of Friend virus-transformed proerythroid cells.

In marked contrast to the above results TPA repeatedly induced differentiation of RV-133 and RV-187 cells (Fig. 1). It had no inhibitory or additive inductive effects on eight other known inducers of haemoglobin synthesis in these cells. Induction seems to be specific for promoters, as phorbol had no effect. The results presented in Fig. 1 clearly show that 10^{-7} – 10^{-9} M TPA induced differentiation of both virus-transformed proerythroid cell lines, although maximum induction occurred with 10^{-4} M TPA. RV-187 cells were more responsive than RV-133 cells to low concentrations of TPA.

Previous studies^{12–14} had shown that both Friend and Rauscher virus-transformed proerythroid cells became committed to differentiate after approximately 48 h of exposure to 1% DMSO. Committed cells will eventually differentiate even after removal of the inducer. To clarify the mechanism of action of tumour promoters, it was important to determine whether cells became committed to differentiate following exposure to TPA. The kinetics of differentiation and commitment to differentiation of RV-133 cells treated with 10^{-4} M TPA or 1% DMSO were determined¹² as described in Fig. 2. As expected, TPA induced differentiation of RV-133 cells (Fig. 2). Surprisingly, the results also indicated that RV-133 cells become committed to differentiation very soon after exposure to TPA. Nearly all of the cells that were eventually measured as differentiated cells became committed to this process within 24 h of exposure to TPA, with maximum levels of commitment to differentiation occurring within 48 h of exposure. Extrapolation of the data suggests that only a few hours' exposure to TPA is required to initiate commitment. In contrast, commitment induced by exposure to DMSO was detected after approximately 48 h, and maximum levels of commitment occurred only after 96 h. Commitment induced by TPA was, therefore, much more rapid than that by DMSO or any other known inducer^{12–14} of differentiation in virus-transformed proerythroid cells. Additional data (manuscript in preparation) now show that the early events during induction of differentiation by TPA are different from those with DMSO. Haemoglobinised cells in both TPA- and DMSO-treated RV-133 cultures were first detected after approximately 60 h of exposure to either inducer (Fig. 2). These results indicate that

Table 1 % Inhibition of differentiation of CI 745 by TPA

Inducers	TPA concentration (M)				
	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
None	87	63	38	0	0
1% DMSO	Toxic	28	21	14	0
0.1% Hypoxanthine	Toxic	42	42	28	14
1% Acetamide	Toxic	28	28	28	14

Cells were cultivated in microtitre plates in the presence of the appropriate concentration of TPA with or without inducers. After 5 days incubation the degree of differentiation (haemoglobin synthesis) was found by determining the number of benzidine-reactive (bz⁺) cells out of about 200–500 cells counted¹². The % inhibition of differentiation relative to control levels was then calculated. The % bz⁺ cells in controls without TPA were: spontaneous, 8%; 1% DMSO, 80%; 0.1% hypoxanthine, 80%; 1% acetamide, 80%.

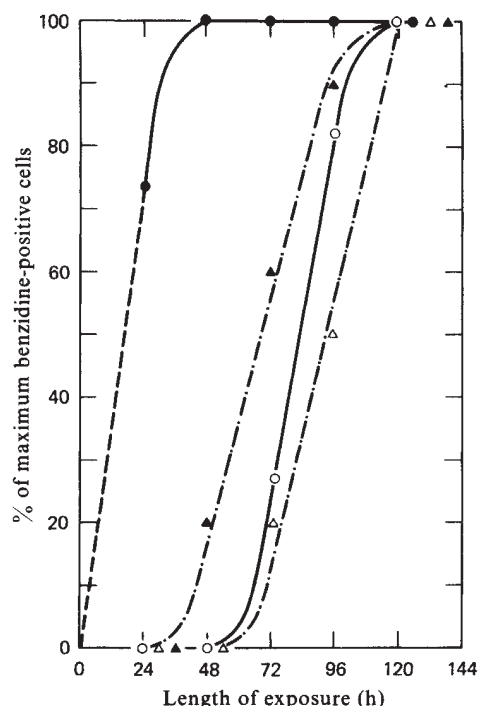


Fig. 2 Kinetics of commitment to and induction of differentiation of RV-133 cells exposed to TPA or DMSO. Cells were cultivated for varying lengths of time in the presence of 10^{-4} M TPA (○, ●) or 1% DMSO (△, ▲). At intervals, inducers were removed and cultures were either treated with benzidine reagent and assayed immediately for benzidine-reactive cells (open symbols) or fresh medium was added and the cultures were incubated for a total of 120 h, at which time they were assayed for benzidine-reactive cells (closed symbols). The ordinate is the % of benzidine-positive cells after varying lengths of exposure to inducer relative to the % of benzidine-positive cells after 120 h exposure to inducers.

although the time required for commitment to differentiation may differ, that for expression of globin genes and haem synthesis is apparently the same regardless of inducer.

We have suggested that RV-133 and RV-187 are physiologically different from Cl 745 and that these differences may reflect their different developmental histories^{12,15}. Our present data agree with this concept and further suggest that the regulatory and/or biochemical steps for haemoglobin synthesis which are arrested in these cells are also different. We have indicated here that tumour promoters can have different and completely opposite effects on virus-transformed proerythroid cells *in vitro* and that the effect may be dependent on the physiological state of the particular cell line under investigation. Therefore, caution must be used in extrapolating certain effects observed *in vitro* for tumour promoters with those as yet unknown functions resulting in promotion of oncogenesis *in vivo*.

TPA and phorbol were obtained from Dr P. Borchert.

Note added in proof: We have now found that the tumour promoters phorbol-12,13-dibenzoate, phorbol-12,13-didecanoate, and anthralin also have opposing effects on erythroid differentiation in Cl 745 and RV-133 cells.

RAYMOND M. MIAO
A. HOWARD FIELDSTEEL
DOUGLAS W. FODGE

Life Sciences Division,
SRI International,
Menlo Park, California 94025

Received 29 March; accepted 17 May 1978.

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3T3 variants lacking receptors for epidermal growth factor are susceptible to transformation by Kirsten sarcoma virus

TODARO *et al.*¹ have reported that transformation of established cell lines by murine (Kirsten, Molony) and feline sarcoma viruses resulted in the loss of receptors for the polypeptide mitogen epidermal growth factor (EGF). The phenomenon showed several forms of specificity; (1) transformation by other viruses such as avian arcoma viruses, polyoma virus or SV40 did not lead to the loss of EGF receptors, (2) transformation by Kirsten murine sarcoma virus did not lead to the loss of cellular receptors for another mitogenic peptide known as multiplication stimulating activity (MSA)², and (3) in a related observation, human fibrosarcoma cell lines had high levels of EGF receptors but low levels of MSA receptors². From these observations Todaro *et al.*² formulated the following hypothesis: "Cell growth in an organism is controlled in part by the particular display of specific growth factor receptors responding to hormones produced by other cells. In the normal situation those cells that produce a specific growth factor do not respond to it. But the inappropriate production by a target cell of an active hormone, that it also has receptors for, may be sufficient to serve as a stimulus for cell division. Persistent uncontrolled production of the growth factor may then serve as a continuous endogenous stimulus and lead to inappropriate cell growth." As one alternative, this group has suggested that transformation by murine sarcoma virus might lead to "the production of endogenous EGF or a related substance that binds to EGF receptors"¹; and have in fact suggested that "the sarcoma genes code for an EGF related substance"¹. This model suggests that the phenotypic expression of viral transformation can be broken at two possible points. If the sarcoma gene product is altered to such a degree that it is unable to bind to the appropriate mitogen receptor, the cell will remain untransformed. Alternatively, untransformed cell lines lacking specific mitogen receptors should not show phenotypic transformation (that is, focus formation) when exposed to the appropriate virus, as the sarcoma gene product produced as a consequence of the transformation event will be unable to stimulate cell division if the appropriate receptor is absent. We have recently isolated and characterised variant Swiss 3T3 cell lines which grow to low cell densities, do not show a mitogenic response to added EGF, do not bind labelled EGF, even at concentrations of hormone 10-fold greater than required for receptor saturation by the parent cell line, and respond to a variety of other mitogens³. According to the model proposed by Todaro *et al.*¹ transformation of these cells by a murine sarcoma virus should not lead to the characteristic alteration in cell density, as the EGF receptor postulated to be 'responsive' to the murine sarcoma gene product is not present. Our studies, however, suggest these EGF receptorless variants are as susceptible to transformation by Kirsten sarcoma virus as the parent 3T3 line.

Flasks of the Swiss 3T3 parental cell line and the three variants NR1, NR4 and NR6 were coded in one laboratory

Table 1 Transformation focus assay titre of Kirsten sarcoma virus on 3T3 and variant cells lacking the EGF receptor

Cell line	Foci per ml
3T3	6×10^4
3T3 (NR1)	13×10^4
3T3 (NR4)	3×10^4
3T3 (NR6)	8×10^4

(H.R.H., R.M.P.) and transferred to the second laboratory (V.K.) for transformation analysis. After the data for the quantitative transformation assay were obtained the code was broken. Mass transformation occurred for all four cell lines at the same dilution of the virus stock (Fig. 1). When the viral transformation titre was determined by counting foci, all four cell lines were essentially equivalent targets for transformation by Kirsten sarcoma virus (Fig. 1, Table 1).

Nuclear receptors have been demonstrated for both polypeptide hormones⁵ and growth factors⁶. It might be argued that viral transformation is mediated by internal nuclear EGF receptors present in the nonresponder variant, but undetectable in our whole-cell binding assays. To examine this possibility we assayed for nuclear EGF receptors in preparations of membrane fragments prepared from both 3T3 cells and one of the nonresponder variants (NR6). Data are summarised in Table 2. NR6 cells have no membrane-associated 3T3 receptors detectable in this assay. Interestingly, although we find that whole 3T3 cells bind about 1 ng of EGF per mg of total cellular proteins, membrane fragments bind considerably more EGF. A large pool of EGF receptors may exist on internal membrane structures which are present in the pellet prepared by this procedure.

Another class of explanations involves the production by the variant cells of inactive substances that block surface EGF receptors, or bind to and/or degrade externally provided EGF. Such a molecule would have to have an extremely high affinity for the EGF receptor, as the binding assay³ is carried out on washed cells at 4 °C, where minimal biosynthetic activity will occur. One must thus postulate that the variant produces an antagonist with a much greater receptor affinity than EGF. To detect such a molecule, extracts of 3T3 and NR6 cells were prepared according to the initial steps of the EGF purification procedure⁷, and examined for inhibition of binding of ¹²⁵I-EGF to 3T3 cells. Fifteen plates of each cell line were washed with cold buffer [0.01 M Tris, pH 7.2, 0.15 M NaCl] and quickly scraped, in a minimal volume, from the plates. Cells were quickly collected by centrifugation and homogenised in 5 ml of

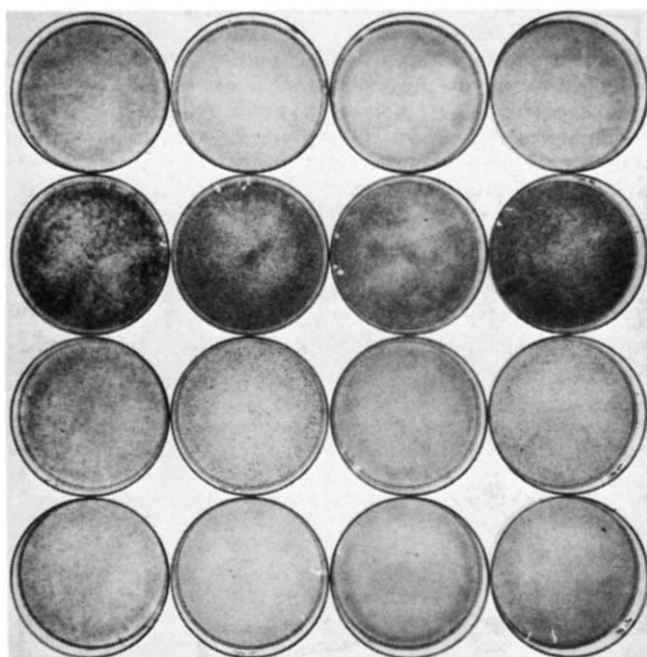


Fig. 1 Transformation by Kirsten sarcoma virus of 3T3 and variant cells lacking EGF receptor. Plates (60 mm) were treated with 0.1% gelatin and seeded with 2×10^5 cells in Dulbecco's medium with 10% heat-inactivated fetal calf serum. After 24 h incubation with polybrene ($10 \mu\text{g ml}^{-1}$) virus dilutions (10-fold) were added to cultures. Virus stocks were prepared from line 58967 as described by Roy-Burman and Klement⁴. When cultures were 90% confluent they were switched to medium containing heat-inactivated postnatal calf serum and 1% DMSO for better distinction of foci. Plates were fixed and stained with Giemsa. Rows from left to right; 3T3, 3T3(NR1), 3T3(NR4), 3T3(NR6). Rows from top to bottom; control (no virus), 10^1 dilution, 10^2 dilution, 10^3 dilution.

0.05 M acetic acid. The supernatants from a centrifugation at 100,000g were dialysed against 0.05 M acetic acid, then lyophilised. The extracts were resuspended in 0.5 ml of 0.05 M HCl, then dialysed overnight against binding assay buffer³. Triplicate 0.1-ml samples were used as potential inhibitors of ¹²⁵I-EGF binding to 3T3 cells. No inhibitory activity was present in either extract.

Table 2 ¹²⁵I-EGF binding to 3T3 and NR6 cells

	Total c.p.m.	Nonspecific c.p.m.	Specific binding c.p.m.	ng EGF per mg protein
3T3 whole cells	40,581	15,728	$24,094 \pm 1,668$	0.85
	42,055	18,721		
3T3 membrane pellet	$22,120 \pm 3,208$	$10,010 \pm 1,456$	$12,110 \pm 1,761$	3.67
NR6 whole cells	16,269	16,491	0	0
	16,878	17,968		
NR6 membrane pellet	$5,420 \pm 492$	$5,458 \pm 693$	0	0

Binding on whole cells was done on ice on 35-mm plates for 90 min, using 66.8 ng per ml ¹²⁵I-EGF (specific activity 96,600 c.p.m. ng⁻¹). Nonspecific binding was determined in the presence of 400 ng per ml of unlabelled EGF. Assays were carried out in duplicate as described³; both values are reported. Specific binding is the difference between the averages $\pm$ s.e.m. Binding to 30 μ l of membrane suspension was assayed on ice using 125 ng per ml ¹²⁵I-EGF (specific activity 236,000 c.p.m. ng⁻¹). Nonspecific binding was determined in the presence of 9.1 μ g per ml of unlabelled EGF. Cells from a confluent 100-mm culture dish were homogenised in 2 ml of binding medium (saline A⁵ containing 0.1% bovine serum albumin). The homogenate was centrifuged at 700g for 10 min. The membrane pellet was resuspended in 0.3 ml of binding medium and 30 μ l of the suspended membranes were added to the tip of a microfuge tube. Membranes were incubated for 3 h on ice with ¹²⁵I-EGF (125 ng ml⁻¹) in a total volume of 40 μ l. Nonspecific binding was measured in the presence of excess unlabelled EGF. Pellets were then washed three times by centrifugation and the tip of the tube (containing the membrane pellet) was assayed for bound ¹²⁵I-EGF. Assays were carried out in quadruplicate. Specific binding is the difference between the means $\pm$ s.e.m. Protein in cells and membranes, solubilised in 0.5 M NaOH, was determined by the method of Lowry⁶. NR6 cells: 263 μ g per 35-mm plate, 13.8 μ g per 30 μ l membranes. 3T3 cells: 293 μ g per 35-mm plate, 31.2 μ g per 30 μ l membranes.

Our data demonstrate that 3T3 variant cells with no functional EGF receptors are still subject to transformation by Kirsten sarcoma virus. Although other alternatives are possible, the most parsimonious interpretation of our data suggests that stimulation of mitogenesis by the action of a sarcoma gene product on EGF receptors is not the basis for uncontrolled growth as a consequence of transformation by murine sarcoma viruses.

We thank A. Stanley for technical assistance. This work was supported by contract EY-76-C-03-0012 between DOE and the University of California (H.R.H.), NIH grant CA 15276 (H.R.H.) and PHS contract NO1-CP 53500 from the NCI Virus Cancer Program (V.K.). R.M.P. was supported by NCI training grant T32-CA-09056.

REBECCA M. PRUSS
HARVEY R. HERSCHMAN*

Department of Biological Chemistry and
Laboratory of Nuclear Medicine,
UCLA School of Medicine,
Los Angeles, California 90024

VACLAV KLEMENT

Departments of Pediatrics and Microbiology,
School of Medicine, University of Southern California,
2025 Zonal Avenue
Los Angeles, California 90024

Received 7 September 1977; accepted 30 May 1978.

*To whom correspondence should be addressed.

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Effect of dibutyryl cyclic AMP on intracellular levels of avian sarcoma virus specific RNA

AVIAN sarcoma viruses readily induce neoplastic transformation of fibroblasts in culture. Genetic and biochemical studies suggest that a single gene, generally referred to as *src*, is responsible for transformation^{1–3}. Nucleic acid hybridisation data, with purified *src* nucleotide sequences, have revealed that the majority of avian and mammalian RNA tumour viruses that are capable of transforming fibroblasts contain a species-specific *src* gene in viral as well as their host genomes^{4–7}. The nature and function of the *src* gene product and the mechanism by which it transforms fibroblasts remain to be elucidated. Indirect evidence, derived from using conditional mutants in the transforming (*src*) gene, suggests that the *src* gene product acts directly on the surface, modulating assembly of the cells⁸. Cyclic AMP seems to be involved in the growth control of a variety of cells⁹. From studies with *ts* mutants in the *src* gene, Pastan *et al.*¹⁰ have proposed that the *src* gene product modifies adenylate cyclase activity directly or indirectly, leading to a decreased level of intracellular cyclic AMP, which in turn enhances the growth rate and alters other morphological features. Here we present evidence that led us to conclude that cyclic AMP, in the presence of the *src* gene product, in fact, decreases the intracellular level of virus-specific RNA.

Primary cultures of chick embryo fibroblasts, grown in medium 199 (ref. 11), were infected with avian sarcoma virus at a multiplicity of 0.05 to 0.1. We routinely observed that in about 10–12 d the majority of cells were transformed, as shown by the appearance of cells with round morphology. Virus-

specific RNA was monitored by nucleic acid hybridisation. Radiolabelled complementary DNA (cDNA) to virion RNA was synthesised in an endogenous reverse transcriptase reaction as described by Garapin *et al.*¹², except that unlabelled substrates were present at 1 mM and ³H-dTTP was present at 0.1 mM. The ³H-cDNA product is fairly representative of the genome as determined by standard saturation experiments. All ³H-cDNAs (B77, Pr-B, SR-A) were prepared in a similar way. RNA from transformed cells was extracted by the method of Glison *et al.*¹³, hybridised to virus-specific ³H-labelled cDNA, and the extent of hybridisation determined using single strand-specific S1 nuclease as described previously¹⁴. In agreement with published data^{15,16}, a *C_t*_{1/2} between 5 and 10 was obtained, indicating that about 0.2–0.4% of total cellular RNA is virus specific.

Preliminary experiments indicated that maximum depression (75–80%) in the levels of virus-specific RNA was observed after treatment for 8 h with 250 $\mu\text{g ml}^{-1}$ *N*⁶-*N*²-dibutyryl cyclic AMP. Time course experiments revealed that as early as 2 h after treatment with dibutyryl cyclic AMP, the RNA levels started to decline and the effect reached a maximum between 8–24 h (Fig. 1a). Prolonged treatment did not result in any further decrease in the transcription of virus-specific RNA. In different experiments approximately three- to fourfold reduction in the *C_t*_{1/2} was observed, indicating that in cultures treated with dibutyryl cyclic AMP only 20–25% of viral RNA compared with untreated control cells was present (Fig. 1a). Removal of dibutyryl cyclic AMP from cultures, 8 h after treatment, completely reversed suppression in less than 8–16 h,

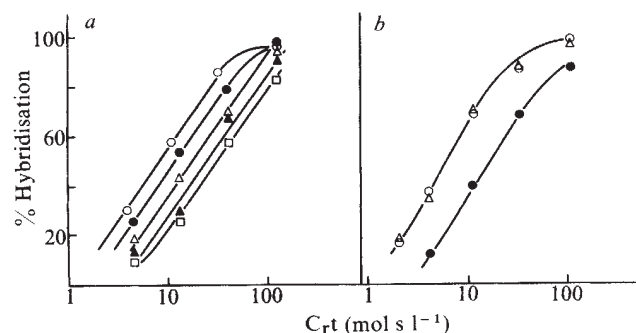


Fig. 1 Effect of dibutyryl cyclic AMP concentration on the transcription of viral RNA. Chick embryo fibroblast cells, transformed by Prague B, grown in monolayers, were treated with 250 $\mu\text{g ml}^{-1}$ dibutyryl cyclic AMP for various periods in the presence of 300 $\mu\text{g ml}^{-1}$ theophylline in medium 199 (Gibco) supplemented with 4% calf serum and 1% dimethyl sulphoxide (DMSO) for 24 h. Cells were collected and the RNA extracted using the CsCl method of Glison *et al.*¹⁴. Briefly, the method consists of lysing cells with 1% sarkosyl, homogenising the lysate and then mixing with 1 g ml^{-1} CsCl. The lysate was layered on a cushion of CsCl (1.35 g ml^{-1}) and centrifuged in a SW50.1 or a SW41 rotor for 14–16 h at 30,000 r.p.m. and 20 °C. The supernatants were poured off and the translucent pellets resuspended in 100–300 μl 20 mM Tris-HCl pH 8.1, and 10 mM EDTA. Virus-specific RNA was monitored by hybridisation to ³H-labelled cDNA as described previously^{15,16}. RNA concentrations were varied, keeping the time of hybridisation constant. The extent of hybridisation was determined using single-stranded specific S1 nuclease¹⁶. Unless otherwise stated, hybridisations were carried out in a volume of 40 μl containing 800–1,000 c.p.m. ³H-cDNA (specific activity 20×10^6 c.p.m. μg^{-1}), 0.6 M NaCl, 10 mM Tris-HCl, pH 8.1, and 3 mM EDTA for 15–20 h at 68 °C. Pr-B cDNA was annealed to 85–88% using Pr-B RNA, which was normalised to 100% in computing *C_t* values. B77 cDNA also reacted to about 80–82% with Pr-B RNA. No differences in the results were observed whichever probe we used. *a*, Untreated control cells (○) or cells treated with dibutyryl cyclic AMP for 45 min (○), 2 h (●), 4 h (△), 8 h (▲) or 24 h (□). *b*, Transformed cells untreated (○) or cells treated with dibutyryl cyclic AMP for 8 h (●) and then removed for 16 h (△).

as shown by the same $C_{rt1/2}$ (Fig. 1b). There was a corresponding decrease in the levels of virus released into the medium after treatment with dibutyryl cyclic AMP (data not shown). Similar results were obtained with different strains of avian sarcoma virus, including B77 and Prague C (subgroup C), Prague B (subgroup B) and Schmidt-Ruppin strain A (subgroup A).

Our experiments were carried out using chick embryo cells transformed by avian sarcoma virus which contains the *src* gene. When similar experiments were carried out using transformation-defective (*td* or *src* negative) B77 virus-infected cells, dibutyryl cyclic AMP did not affect the levels of viral RNA (Fig. 2b). Neither increased concentrations nor prolonged exposure to dibutyryl cyclic AMP resulted in any suppression of RNA levels. These results suggest that the *src* gene product is required for the dibutyryl cyclic AMP-mediated effect on viral RNA.

The requirement for the *src* gene product in the observed effect of dibutyryl cyclic AMP was confirmed by the following experiment. Chick embryo fibroblasts were infected with a *ts* mutant (*tsNY68*) of Schmidt-Ruppin strain A, which had a temperature-sensitive lesion in the *src* gene² or its parent (SR-A) at 36°C. When the cultures were fully transformed, they were shifted to 41°C or maintained at 36°C and then treated with 250 $\mu\text{g ml}^{-1}$ dibutyryl cyclic AMP for 24 h. Total cellular RNA were extracted and assayed for virus-specific transcripts. In complete agreement with the results obtained with B77 *td*-infected cells, virus-specific RNA was specifically reduced in cells treated with dibutyryl cyclic AMP only at the permissive temperature, but not at the restrictive temperature at which the *src* gene product is inactive (Table 1). By contrast, a fourfold reduction in the $C_{rt1/2}$ was observed at both temperatures in SR-infected cells which had been treated with dibutyryl cyclic AMP (Table 1). These results strongly suggest that a functional *src* gene product is absolutely vital for the observed effect of dibutyryl cyclic AMP on the levels of virus-specific RNA.

In all the above experiments ³H-labelled DNA complementary to a large portion of the genomic RNA was used to quantify virus-specific RNA. As the *src* gene product is essential for transformation, we asked whether *src*-specific RNA synthesis was more directly affected by dibutyryl cyclic AMP. To examine the levels of *src*-specific RNA in cultures treated with dibutyryl cyclic AMP, we used sarcoma-specific cDNA (cDNA_{src}) prepared as described⁴. The data presented in Fig. 3 clearly show that total virus-specific RNA was suppressed (as expected) about fourfold, but that *src* RNA synthesis was reduced by at least nine- to 10-fold. For example, in treated controls a $C_{rt1/2}$ of approximately 10 was obtained when total cDNA or cDNA_{src} were used, whereas in cells treated with dibutyryl cyclic AMP the $C_{rt1/2}$ s were 50 and 120, respectively, indicating a reduction of about 80–90% in the levels of virus-specific RNA and *src* RNA. In different experiments we always observed much more reduction in *src*-specific RNA than total viral RNA. The significance of this differential effect is not known.

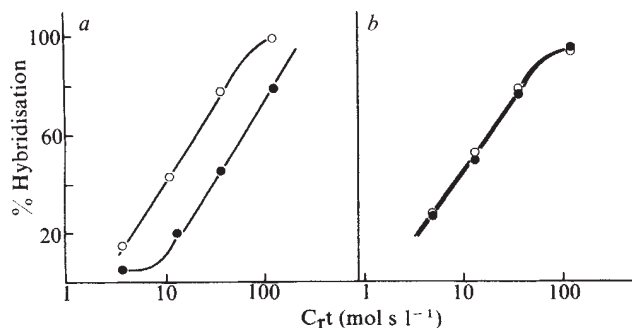


Fig. 2 Effect of dibutyryl cyclic AMP on B77 *td*-infected cells. Chick embryo fibroblasts grown in medium 199 without DMSO, infected by either avian sarcoma virus strain B77 (a), or its transformation-defective deletion mutant (b), were treated with 250 $\mu\text{g ml}^{-1}$ dibutyryl cyclic AMP for 24 h. Total cellular RNA was extracted and analysed for virus-specific sequences using B77 cDNA. In B77 *td*-infected cells, the levels of virus-specific RNA was about two- to threefold less than in the wild type. Addition of DMSO to the growth medium only slightly elevated these levels and the effect of dibutyryl cyclic AMP on transcription of RNA in the presence or absence of DMSO remained the same. Note that treatment, with dibutyryl cyclic AMP, of B-transformed cells, which were passaged several times (6–8 passages), did not result in the expected decrease in the levels of virus-specific RNA. This might be due to segregation of *td* particles, because the cultures kept producing virus even though the cells appeared to be more fibroblastic and flattened. The high frequency of segregation of avian sarcoma viruses, especially B77, is a well known phenomenon¹⁹. a, ASV-transformed control cells (○) or cells treated with dibutyryl cyclic AMP (●). b, B77 *td*-infected control cells (○) or cells treated with dibutyryl cyclic AMP (●).

The results obtained with transformation-defective mutants strongly suggest that the *src* gene product is essential for the observed reversion of transformed cells and suppression of viral RNA by dibutyryl cyclic AMP. Whether dibutyryl cyclic AMP is directly interacting with the *src* gene product in exerting its effect is not known. Two groups have independently postulated that the *src* gene product alters surface properties of cells by interacting with membrane associated polypeptides^{8,10}.

The results presented above can be interpreted to mean that dibutyryl cyclic AMP, in the presence of the *src* gene product, suppresses virus-specific RNA transcription, although other possibilities, such as increased rate of RNA turnover, have not been excluded. We do not, at present, know the mechanism by which dibutyryl cyclic AMP exerts its effect on virus-specific RNA. It is possible that dibutyryl cyclic AMP and the *src* protein react together or independently with a membrane component, thereby activating a specific repressor which causes suppression of transcription of viral RNA or a specific nuclease that destroys viral RNA. As cyclic AMP activates protein kinases in a number of systems, it is also possible that such a

Table 1 Effect of dibutyryl cyclic AMP on *tsNY68* at permissive and non-permissive temperatures

	Total virus-specific RNA				Mutant (<i>tsNY68</i>)			
	Parent		Mutant (<i>tsNY68</i>)		Parent		Mutant (<i>tsNY68</i>)	
	36°C	41°C	36°C	41°C	36°C	41°C	36°C	41°C
	$C_{rt1/2}$ (mol s l^{-1})	% Virus-specific RNA	$C_{rt1/2}$ (mol s l^{-1})	% Virus-specific RNA	$C_{rt1/2}$ (mol s l^{-1})	% Virus-specific RNA	$C_{rt1/2}$ (mol s l^{-1})	% Virus-specific RNA
Control	10	0.2	16	0.12	11	0.2	18	0.11
Dibutyryl cyclic AMP	40	0.05	60	0.03	40	0.05	18	0.11

Chick embryo fibroblasts were infected by Schmidt-Ruppin strain of avian sarcoma virus with the wild-type, or a mutant strain with a lesion in the *src* gene, at 36°C. When the cells were completely transformed, cells were kept at 36°C or shifted up to 41°C for 4–5 h; the cells were either treated with 250 $\mu\text{g ml}^{-1}$ dibutyryl cyclic AMP for 24 h or left untreated. RNA was extracted from all the cultures and assayed for virus-specific RNA using Schmidt-Ruppin viral cDNA. The amount of virus-specific RNA was computed from the $C_{rt1/2}$ values.

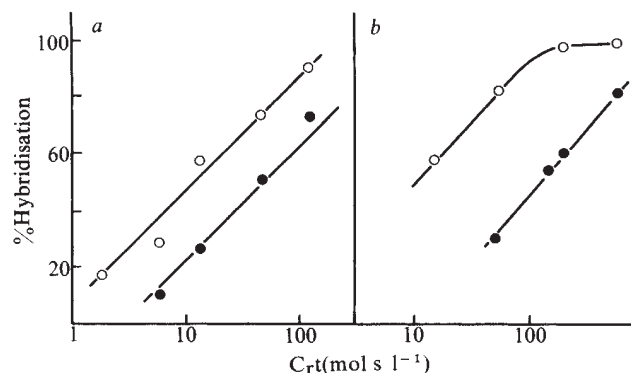


Fig. 3 Suppression of *src*-specific RNA by dibutyryl cyclic AMP. Prague B-transformed cells were treated with 250 μ g ml⁻¹ dibutyryl cyclic AMP for 8 h. RNA was extracted from untreated and treated cultures using the CsCl method and hybridised to either ³H-labelled DNA complementary to the entire sarcoma virus genome (a) or to ³H-labelled *src*-specific DNA sequence (b). Approximately 1,000 c.p.m. representative cDNA (specific activity 20×10^6 c.p.m. μ g⁻¹) or 600 c.p.m. (specific activity 20×10^6 c.p.m. μ g⁻¹) cDNA_{src} were used in each hybridisation reaction. a, Control cells (O) or cells treated with dibutyryl cyclic AMP (●). b, cDNA_{src} hybridised to control (O) or dibutyryl cyclic AMP RNA (●). With cDNA_{B77} and cDNA_{Pr-B} the reactions reached a maximum at 82 and 89%, respectively, which were taken as 100% in computing the $C_{rt1/2}$ values. In several experiments we have observed the same effect whichever probe we used. cDNA_{src} was hybridised to greater than 95% with B77 or Pr-B RNA.

kinase phosphorylates the *src* gene product or a secondary component which then acts as a virus-specific repressor or a nuclease. These possibilities are being tested.

We thank Dr H. Hanafusa, Rockefeller University, for supplying virus stocks, and Drs B. F. Erlanger and H. S. Ginsberg for comments on the manuscript. This work was supported by grant CA19152 awarded by the National Cancer Institute.

RAMAREDDY V. GUNTAKA
AMY J. WEINER

Department of Microbiology,
College of Physicians and Surgeons,
Columbia University,
New York, New York 10032

Received 30 September 1977; accepted 8 May 1978.

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Oestrogen modulates progesterin receptor concentrations in some rat brain regions but not in others

It is now well established that progesterone can either facilitate or inhibit the effects of oestrogen on gonadotrophin release and behaviour¹. Whether facilitation or inhibition is observed depends primarily on the time interval between exposure to oestrogen and progesterone, but the mechanism by which progesterone exerts these effects remains unknown. Attempts to demonstrate progesterone receptor sites within the brain and pituitary have given equivocal results^{2–10}, leading to speculation that the central effects of progesterone may be mediated through a mechanism fundamentally different from that found in peripheral tissues^{1,2,6}. More recently, however, several studies have suggested that oestrogen-inducible progesterin receptor systems similar to those found in peripheral progesterone target tissues can be identified in the brain and pituitary if sufficiently sensitive and specific experimental procedures are used^{11–14}. We summarise here work from our laboratory which indicates that, in the rat brain, there are in fact two anatomically distinct progesterin receptor systems, one of which differs strikingly from the systems identified previously in neural and non-neural tissues in that it is apparently insensitive to oestrogen.

Progesterin receptors labelled with the potent synthetic progesterin ³H-R5020 (17 α ,21-dimethyl-19-norpregna-4,9-diene-3,20-dione) in soluble cytoplasmic ('cytosol') fractions from rat uterus, pituitary and hypothalamus plus preoptic area were found to sediment at ~7S on sucrose density gradients containing 10% (v/v) glycerol (Fig. 1a–c), in agreement with previous reports^{2,12,13,15}. The properties of these receptor complexes—their sedimentation coefficients, saturability in the presence of 10⁻⁶M unlabelled R5020, and increased cytosol concentrations after oestrogen treatment—clearly distinguished them from the large quantities of ³H-R5020 binding present in serum (Fig. 1f). In the midbrain and cerebral cortex, however, binding was observed which sedimented at ~7S and was saturated by 10⁻⁶M R5020, but unlike the receptor complexes identified above, was present in equal concentrations in cytosols from untreated and oestrogen-primed animals (Fig. 1d, e).

Qualitatively similar results were obtained using gel filtration on Sephadex LH-20 (ref. 18). Scatchard¹⁹ plots constructed with this technique in cytosols from midbrain, cortex, hypothalamus–preoptic area, pituitary and uterus were rectilinear, indicating the presence of high-affinity ($K_d \sim 0.3$ nM for R5020) limited-capacity binding sites (Fig. 2 and Table 1). Following oestrogen treatment, the number of available high-affinity sites increased sharply in hypothalamus–preoptic area, pituitary and uterus cytosols, but did not change in cytosols from midbrain and cerebral cortex. Consistent with the density gradient sedimentation data, no high-affinity limited-capacity binding was observed in serum (data not shown).

Further studies with the Sephadex LH-20 technique demonstrated that in each of the tissue cytosols studied, from both oestrogen primed and nonprimed ovariectomised female rats, the high affinity ³H-R5020 binding sites possess several properties characteristic of cytoplasmic progesterin receptors in addition to their sedimentation coefficients and affinities for ³H-R5020. (1) They are apparently progesterin specific: binding of ³H-R5020 is inhibited in the presence of 25-fold molar excesses of unlabelled progesterone, or of the synthetic progestins *d*-norgestrel, norethindrone or medroxyprogesterone acetate, but is unaffected by similar concentrations of dexamethasone, corticosterone, testosterone or 17 β -oestradiol. (2) They are at least partly composed of protein, as binding is destroyed by incubation with pronase. (3) Free sulphhydryl groups seem to be essential for binding activity, as

no binding is observed if dithiothreitol is omitted from the homogenisation buffer, or if the sulphhydryl blocker, *p*-chloromercuribenzoate, is added to the cytosol fractions. (4) They are extremely thermolabile: incubation at 25°C for 30 min reduces the concentration of high-affinity sites by ~50%. (5) They are precipitated by protamine sulphate in the conditions described by Steggle and King²⁰ for the oestrogen receptor, suggesting that they have several accessible anionic groups.

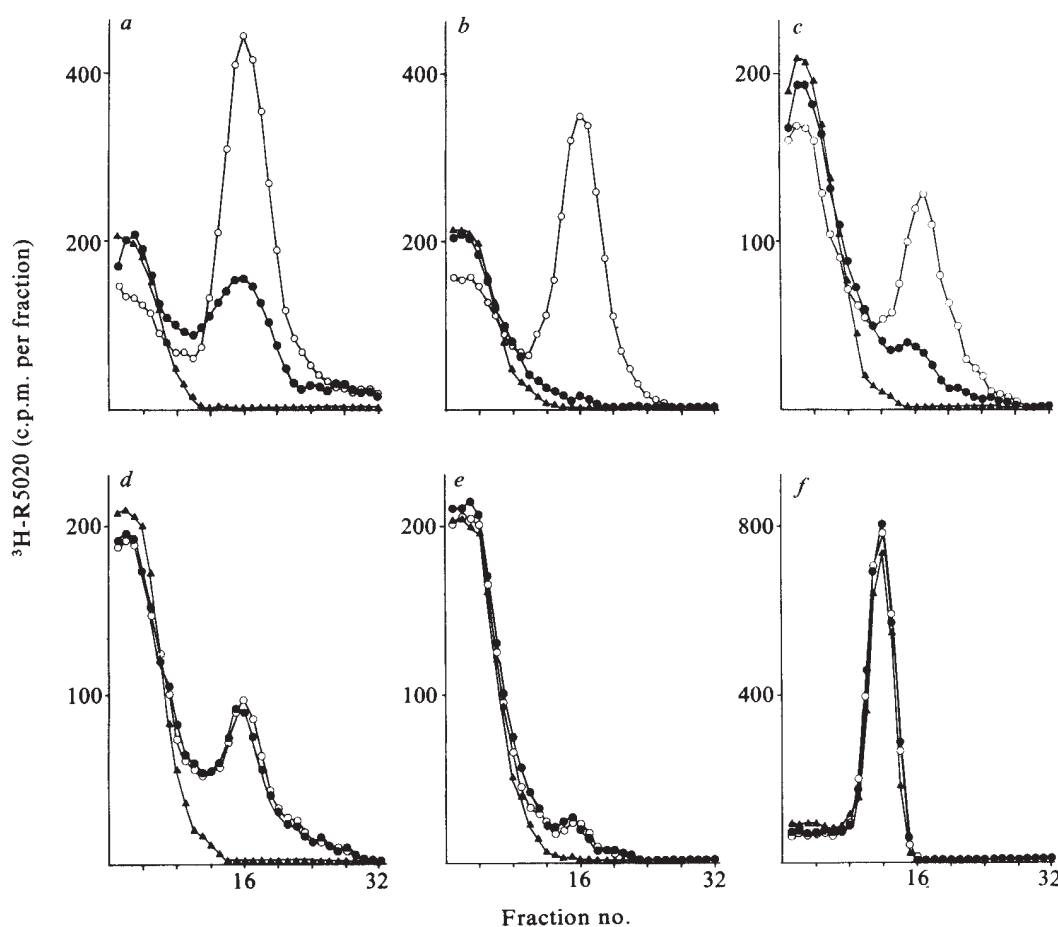
Several conclusions can be drawn from these data about the nature of the progesterin receptor systems in rat brain. The properties of the cytoplasmic high affinity ³H-R5020 binding sites strongly suggest that these sites represent progesterin receptor molecules. If so, then it seems clear that these receptors are not confined to the hypothalamus and preoptic area, but are also present in the midbrain and cerebral cortex (a conclusion consistent with the cell nuclear ³H-R5020 uptake studies of Blaustein and Wade¹¹). There is, however, a divergence in sensitivity to oestrogen between the receptor systems present in different brain regions. In the hypothalamus and preoptic area, as in the pituitary and uterus, the receptors are present at low concentrations in the absence of oestrogen, and

increase in number after oestrogen treatment. In contrast, in the midbrain and cerebral cortex, they are present at levels which are not affected by short-term oestrogen treatment.

On the basis of these observations, we propose that the rat brain contains two distinct progesterin receptor systems: one, localised in the hypothalamus and preoptic area, is regulated by oestrogen; the second, which is present in the midbrain and cerebral cortex, is relatively insensitive to elevated plasma oestrogen levels. The extent to which these two systems are represented in other brain structures remains unknown. Preliminary studies suggest that progesterin receptors are also present in the amygdala, hippocampus, caudate-putamen and cerebellum, at levels comparable to those found in the midbrain. In all of these tissues, as in the cortex and midbrain, oestrogen treatment does not affect the concentration of progesterin receptor sites (data not shown).

The existence of an oestrogen-sensitive progesterin receptor system in the hypothalamus and preoptic area is consistent with reports that progesterone implants in these areas can facilitate or inhibit oestrogen-induced female sexual behaviour^{1,21,22}. The situation with respect to the apparently oestrogen-insensi-

Fig. 1 Sucrose gradient sedimentation of bound ³H-R5020 in serum (f) and soluble cytoplasmic fractions from the uterus (a), pituitary (b), hypothalamus plus preoptic area (c), cerebral cortex (d) and midbrain (e) of adult female Sprague-Dawley rats. All animals were adrenalectomised and ovariectomised 10–14 d before use. They were treated subcutaneously for 3 d with either 15 µg per day of 17β-oestradiol-3-benzoate (EB) in sesame oil, or the oil vehicle alone. On the fourth day blood was taken by cardiac puncture under methoxyflurane anaesthesia. The animals were then killed by perfusion through the left ventricle with ice-cold 6% dextran in 0.85% saline. Tissues were removed onto a chilled frosted glass platform, and the brain dissected as previously described¹⁶. Tissue homogenates were prepared in buffer containing 10 mM Tris, 1.5 mM disodium-EDTA, 1 mM threitol and 10% (v/v) glycerol, adjusted to pH 7.4 with hydrochloric acid (TEGD buffer). The homogenates were centrifuged at 106,500g



(average) for 1 h to obtain clear supernatants. Serum was separated from the clotted blood samples by centrifugation, and diluted 1:10 with TEGD buffer. The tissue supernatants and diluted sera were incubated with 0.4 nM [6,7-³H]R5020 (56.5 Ci mmol⁻¹) for 4 h at 2–4 °C, with or without 10⁻⁶ M unlabelled R5020. Aliquots of the incubates were sedimented through 5–20% (w/v) linear sucrose gradients in TEGD buffer for 20 h at 1 °C, at an average of 210,000g. The gradients were collected by tube puncture in 10-drop (~0.16 ml) fractions, and counted at an efficiency of 32%. ○, EB treated + 0.4 nM ³H-R5020; ●, oil treated + 0.4 nM ³H-R5020; ▲, EB treated + 0.4 nM ³H-R5020 and 10⁻⁶ M unlabelled R5020. The quantities of soluble protein applied to each gradient, measured by the method of Lowry *et al.*¹⁷, were as follows: serum, EB 1.44 mg, oil 1.53 mg; uterus, EB 0.180 mg, oil 0.170 mg; pituitary, EB 0.45 mg, oil 0.54 mg; hypothalamus plus preoptic area, EB 1.14 mg, oil 1.24 mg; midbrain, EB 1.13 mg, oil 1.20 mg; cerebral cortex, EB 1.26 mg, oil 1.29 mg. The sedimentation markers horse heart myoglobin (2.0S), bovine serum albumin (4.6S) and bovine liver catalase (11.3S) sedimented, respectively, in fractions 6, 12 and 26. The discrepancy between the gradients in total recovered radioactivity is due to adsorption of free ³H-R5020 to the cellulose nitrate centrifuge tubes. This adsorption is less pronounced in samples containing high concentrations of binding sites.

Table 1 Equilibrium dissociation constants and saturation capacities for binding of ^3H -R5020 to high-affinity cytosol binding sites

	K_d ($\text{M} \times 10^{10}$)		Saturation binding capacity (fmol per cytosol protein)	
	Oil	EB	Oil	EB
Uterus	3.13 ± 0.47	2.88 ± 0.56	328.8 ± 79.1	$2,270.1 \pm 223.5$
Pituitary	3.15 ± 0.55	2.49 ± 0.30	31.2 ± 4.2	273.4 ± 11.8
Hypothalamus + preoptic area	2.99 ± 0.31	3.08 ± 0.34	15.4 ± 1.9	36.5 ± 7.5
Cerebral cortex	3.69 ± 0.27	3.83 ± 0.25	27.6 ± 4.1	26.5 ± 3.0
Midbrain	3.12 ± 0.33	2.64 ± 0.13	11.2 ± 1.6	11.9 ± 0.6

Equilibrium dissociation constants (K_d s) and saturation capacities for reaction between ^3H -R5020 and high-affinity binding sites in cytosols from sesame oil or oestradiol benzoate (EB; $15 \mu\text{g d}^{-1}$; 3 d) treated adrenalectomised-ovariectomised rats. Data were calculated from Scatchard representations of binding isotherms similar to those in Fig. 2. Values represent the means ($\pm$ s.e.m.) of three observations.

tive sites in other brain regions is less clear. They may be involved in mediating effects of progestins which are not dependent on prior exposure to oestrogen; for example, effects on cortical electroencephalograph patterns²³ or the 'concurrent' type of progestin-induced inhibition of female sexual behaviour²⁴. It is possible that interplay between the oestrogen-sensitive and -insensitive receptor systems may underlie the sequential changes in behavioural and neuroendocrine responsiveness to progesterone which follow oestrogen treatment¹.

We thank Dr C. Martucci, Rockefeller University, for medroxyprogesterone acetate, Dr H. Nash of the Population Council for norethindrone and *d*-norgestrel, Dr J-P. Raynaud,

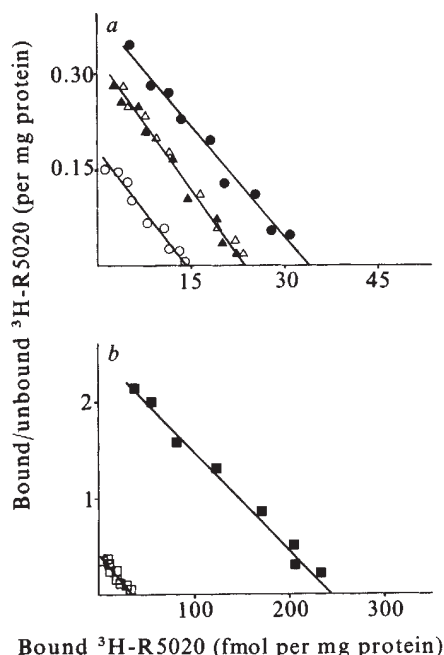
Roussel-Uclaf, for unlabelled and 6,7 ^3H -labelled R5020, and Drs E. Roy and L. C. Krey for helpful comments. This work was supported by grant NS 07080 from the USPHS to B.S.M., by institutional grant RF 70095 from the Rockefeller Foundation, and by a postdoctoral fellowship from the UK SRC to N.J.M.

NEIL J. MACLUSKY
BRUCE S. MCEWEN

The Rockefeller University,
1230 York Avenue,
New York, New York 10021

Received 30 March; accepted 22 May 1978.

Fig. 2 Scatchard plots of ^3H -R5020 binding in cytosols from rat brain, pituitary and uterus. Cytosols were prepared as described in Fig. 1 from tissues of sesame oil or oestradiol benzoate (EB) treated ovariectomised-adrenalectomised female rats, and incubated with a range of ^3H -R5020 concentrations (0.02–3.20 nM) for 4 h at 2–4 °C in the presence or absence of a 50-fold molar excess of unlabelled R5020. Aliquots (250 μl) of each incubate were chromatographed on columns (0.5 $\times$ 7 cm) of Sephadex LH-20 equilibrated with TEGD buffer at 2–4 °C, to separate macromolecular bound and free steroid¹⁸. High-affinity bound ^3H -R5020, obtained as the difference in binding between preparations incubated with and without unlabelled R5020, is plotted by the method of Scatchard¹⁹. *a*, Hypothalamus–preoptic ($\circ$, $\bullet$) and cerebral cortex (Δ , $\blacktriangle$) cytosols; *b*, pituitary cytosols. Open symbols, oil treated; closed symbols, EB treated.



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Direct evidence for presynaptic and postsynaptic dopamine receptors in brain

CENTRAL dopamine (DA)-containing neurones are thought to be involved in several human diseases including Parkinson's disease and schizophrenia, and to act as sites of action of some drugs of abuse. One of these DA systems, the nigrostriatal projection (NSP), has its origin in the substantia nigra (SN), pars compacta and innervates the caudate-putamen (CP). In the SN the dendritic processes of DA neurones have been shown to contain DA and vesicles^{1–3} and there is evidence that DA can be released by cells in the SN^{4–6}. It has therefore been proposed that one mechanism by which the activity of DA neurones is regulated is through the action of dendritically

released DA on hypothesised 'autoreceptors' on DA perikarya or dendrites⁷⁻⁹. Recently, however, this view has been challenged by the observation that DA-sensitive adenylate cyclase, an enzyme thought to be coupled with the DA receptor¹⁰, is located on terminals of the striatonigral or pallidonigral projection^{11,12} rather than on the DA cells themselves¹³⁻¹⁵. Similarly, in the CP where the existence of autoreceptors on DA terminals has been suggested¹⁶, the DA-sensitive adenylate cyclase has been shown to be localised on postsynaptic structures rather than on the DA terminals^{17,18}. An established method for studying the DA receptor involves measuring DA receptor binding with labelled DA agonists or antagonists^{19,20}. To further elucidate the location of DA receptors in the SN and CP, we have examined the effects of selective lesions of the NSP on labelling of DA receptors in these structures. These lesions, which are placed in the axons of the NSP, are known to cause near-complete anterograde and retrograde degeneration of the terminals and cell bodies of this system²¹. The advantage of this lesion technique over previous approaches is that it avoids nonspecific damage of the areas studied. Our results provide direct evidence of presynaptic and postsynaptic dopamine receptors in rat brain.

Male Wistar rats (300–320 g) received unilateral 6-hydroxydopamine (6-OHDA) lesions of the NSP at the level of the lateral hypothalamus. After 25 d, the animals were killed and the striatum and SN were dissected out as previously described²². A small portion of the striatum was taken for tyrosine hydroxylase (TH) measurements²³. The remaining striatum and the SN were frozen on dry ice within 3 min of dissection and assayed for DA receptor binding as described in Table 2. A second group of rats received identical lesions. Twenty-five days later, they were killed and TH was measured in the striatum and SN and glutamic acid decarboxylase (GAD) was measured in the SN²⁴.

As shown in Table 1, in the animals in which DA receptor binding was measured, striatal TH activity was reduced by >90% on the side ipsilateral to the 6-OHDA NSP lesion. Animals in the second group showed a similar decrease in striatal TH. Furthermore, in the latter group TH activity was

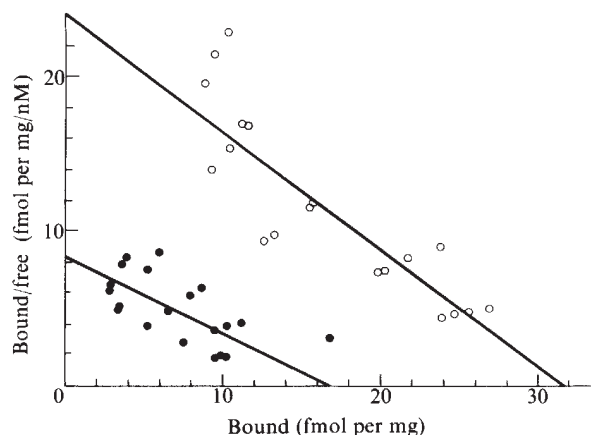


Fig. 1 Scatchard analysis of specific ³H-apomorphine binding to rat striatal homogenates from control unoperated side (○) and from contralateral 6-OHDA-lesioned side (●). Specific binding was that which occurred in buffer minus that in presence of 1 μM (+)-butaclamol. The assays were performed using five concentrations of ³H-apomorphine between 0.5 and 6.0 nM. K_D was 1.31 ± 0.04 nM for control striatum and 2.04 ± 0.14 nM for lesioned striatum (significant at $P < 0.001$, two-tail Student's *t*-test). B_{max} (maximum number of binding sites) was 31.6 ± 0.9 fmol per mg protein for control striatum and 16.8 ± 1.01 fmol per mg protein for lesioned striatum (significant at $P < 0.001$, two-tail Student's *t*-test).

Table 1 Tyrosine hydroxylase and GAD activities in the striatum and substantia nigra after 6-OHDA lesions of the nigrostriatal pathway

Area	Enzyme	Control enzyme activities (nmol per mg protein per h)		Activities after lesion induction (% of control)
		Group 1	Group 2	
Striatum	TH	4.46 ± 0.33 (34)	4.07 ± 0.21 (7)	<10
SN	TH	—	2.29 ± 0.08 (7)	<10
SN	GAD	—	267 ± 13.1 (7)	102

Animals received unilateral injections of 6-OHDA (4 μg per 2 μl) into the nigrostriatal projection. The 6-OHDA was dissolved in 0.15 M NaCl which contained 0.2 mg ml⁻¹ ascorbic acid as antioxidant. The coordinates from stereotaxic zero were A + 5.9, L + 2.1, DV + 1.9 mm. The injection rate was 0.2 μl min⁻¹. Animals received desipramine HCl (25 mg per kg body weight) 30 min before the 6-OHDA injection to prevent damage to noradrenergic neurones. For the assay of TH and GAD, striatal and nigral tissues were homogenised in 50 mM Tris-acetate buffer pH 6.2, containing Triton X-100 (0.20% v/v). For the assay of TH, tyrosine and DMPH₄ cofactor were present at saturating concentrations. All other procedures for the assay of these enzyme activities were as previously described^{23,24}. In the assay of TH a value of twice blank gave 10% of the activity observed in the unlesioned striatum. However, in most cases striatal and SN tissues on the lesioned side showed blank values for TH activity. For example, 32/34 animals in group 1, and 6/7 animals in group 2 gave blank values for TH in the striatum on the lesioned side. The average activity in the 3 remaining animals was 8% of control. Similarly in the SN, 4/7 animals showed blank TH activity and 3 animals showed an average reduction to 9% of control activity. Values represent means ± s.e.m. The number of animals in each group is shown in parentheses.

reduced by >90% in the SN, indicating virtually complete retrograde degeneration of the DA perikarya. In contrast, nigral GAD was not affected by the 6-OHDA lesions, indicating that the GAD-containing terminals of the pallidonigral or striatonigral projections were not significantly damaged by the lesions^{11,12,25}. In the present experiments, the binding of ³H-apomorphine in rat striatum was relatively less than that of ³H-haloperidol and ³H-spiperone (Table 2). This contrasts with previous observations on calf caudate in which binding of the two ligands was essentially identical¹⁹. Note that the 'striatal' samples studied here contained nucleus accumbens, caudate-putamen and globus pallidus. Thus, either species differences or tissue sampling may account for this discrepancy. Our major finding is that the specific binding of ³H-apomorphine to SN membranes was reduced on the lesioned side by 76% (Table 2). Similarly, compared with the control side, NSP lesions reduced ³H-apomorphine binding in the striatum by 56%. The linear regression lines in the Scatchard plot shown in Fig. 1 further indicate that both the affinity of ³H-apomorphine for the DA receptor and the maximum number of binding sites (B_{max}) in NSP-lesioned striatum are significantly reduced compared to control striatum. In marked contrast with the reduction in striatal ³H-apomorphine binding, ³H-spiperone binding and ³H-haloperidol binding were significantly increased by 18% and 22% respectively (Table 2). Elimination of dopaminergic neurones in the SN by the NSP lesions did not alter ³H-spiperone binding in the SN.

Apomorphine has been well characterised as a DA agonist and haloperidol and spiperone as DA antagonists. The validity of using these compounds in the assay of the DA receptor has been demonstrated previously^{19,20,26}. The observation that selective and extensive retrograde loss of DA neurones in the SN reduced ³H-apomorphine binding in this structure by 76% is a direct demonstration of the existence of DA receptors on these DA perikarya. The fact that nigral TH activity was reduced by >90%, whereas nigral ³H-apomorphine binding was reduced by only 76% suggests that there are also DA receptors on nondopaminergic neurones. This is further indicated by the unaltered ³H-spiperone binding in the SN after the NSP lesions. One potential site of the latter type of

Table 2 Binding of ^3H -spiperone, ^3H -apomorphine and ^3H -haloperidol in striatum and SN after 6-OHDA induced lesioning of the nigrostriatal pathway

		^3H -Apomorphine binding		^3H -Haloperidol binding		^3H -Spiperone binding	
		(fmol per mg)	% Control	(fmol per mg)	% Control	(fmol per mg)	% Control
Striatum	Control	11.2 $\pm$ 1.2(11)	100	105 $\pm$ 7.8(6)	100	146.6 $\pm$ 4.9(5)	100
	Lesioned	4.9 $\pm$ 1.3** (8)	44	128 $\pm$ 8.0* (4)	122	172.8 $\pm$ 8.0* (5)	118
Substantia nigra	Control	8.0 $\pm$ 0.8(6)	100	—	—	34.9 $\pm$ 3.7(8)	100
	Lesioned	1.9 $\pm$ 0.8*** (5)	24	—	—	31.7 $\pm$ 3.3 n.s. (8)	91

All comparisons were done by Student's *t*-test (two-tailed). NS, not significant. ^3H -apomorphine was specially prepared and purified by New England Nuclear, Boston. The compound had a specific activity of 14.1 Ci mmol⁻¹ and was 96% pure as determined by thin-layer chromatography. ^3H -haloperidol (IRE Belgique) had a specific activity of 8.4 Ci mmol⁻¹. ^3H -spiperone with specific activity of 26.4 Ci mmol⁻¹ was obtained from NEN. The buffer, used in the preparation of crude brain homogenates and in the binding assays, contained 15 mM Tris-HCl (pH 7.4), 5 mM Na₂EDTA, 1.1 mM ascorbate and 12.5 μM nialamide; (+)-butaclamol (Ayerst) was prepared fresh from a 1 mM ethanol stock by diluting with buffer. Tissues were suspended in 10 volumes of ice-cold buffer and homogenised using a glass homogeniser with a Teflon piston (0.13–0.18 mm clearance, A. H. Thomas). After another addition of buffer, the homogenate was incubated in a water bath at 37 °C for 1 h after which it was divided into 1-ml aliquots and stored at -20 °C. Before use, the crude homogenate was thawed and spun at 40,000g for 15 min at 4 °C in a Sorvall RC2-B refrigerated centrifuge. Final homogenisation was carried out in 3 ml buffer using a Polytron homogeniser (Brinkmann) at a setting of 5.5 (full scale = 10) for 20 s using a PT-10 generator and a 15-ml glass tube (size 20 $\times$ 100 mm) to contain the suspension. Crude homogenate from substantia nigra was prepared in a similar way as described above, except that the substantia nigra tissues were combined into 27 pools (14 for control group, 13 for lesioned group) before homogenisation. For the binding assay, 0.2 ml of membrane suspension (containing 0.2 to 0.3 mg of protein for ^3H -apomorphine and ^3H -haloperidol binding and 0.05 to 0.06 mg of protein for ^3H -spiperone binding) was added to the test tube already containing 0.1 ml (+)-butaclamol (final concentration 100 nM for ^3H -haloperidol binding, 1 μM for ^3H -apomorphine binding, and 0.5 μM for ^3H -spiperone binding), 0.1 ml for buffer and 0.2 ml of ^3H -haloperidol (3 nM) or ^3H -apomorphine (3 nM) or ^3H -spiperone (0.5 nM) and incubated for 30 min. Each experimental value for receptor determination was based on sextuplicate sets of tubes. After the incubation period, 0.5 ml was taken and rapidly filtered through a glass-fibre filter disk (GF/B, 24 mm diameter, Whatman) under negative pressure, and immediately followed by washes of appropriate volumes of buffer¹⁹. After the transfer of the filters and the addition of 8 ml of Aquasol to scintillation vials the radioactivity was monitored by a Packard Tri-CARB (model 3380) liquid scintillation counter.

**P* < 0.05 compared to contralateral control side.

***P* < 0.01.

****P* < 0.001.

binding may be adenylate cyclase-linked DA receptors on the terminals of striatal or pallidal afferents to the SN^{11,12,14}. It could be argued that the decreased ^3H -apomorphine binding in the SN might be due to compensatory changes in DA receptors on these terminals, but this is unlikely because stimulation of adenylate cyclase by DA is not altered by 6-OHDA lesions of the SN^{13,15}.

There have been many reports of enhanced behavioural effects of directly-acting DA agonists such as apomorphine after lesions of the NSP or after chronic treatment with neuroleptics^{27–29}. It is known^{30–32} that these treatments produce an increase in the number of striatal DA receptors assayed with ^3H -haloperidol. Our results confirm the increase in striatal ^3H -haloperidol and ^3H -spiperone binding after lesions of the NSP, and this supports the possibility that this increase may be related to the enhanced behavioural effects of DA agonists after NSP lesions. In contrast to the elevated binding of ^3H -haloperidol and ^3H -spiperone, ^3H -apomorphine binding was significantly reduced in striatal tissue ipsilateral to the NSP lesions. The ^3H -apomorphine data provide direct evidence, therefore, for the presence of DA receptors on DA terminals or axons in the striatum, the existence of which has previously been inferred by other methods—including observations of the *in vivo*¹⁶ and *in vitro*^{33–35} effects of apomorphine and neuroleptics on DA synthesis in the striatum. There is also behavioural evidence for a presynaptic action of low doses of apomorphine^{36,37}; this is consistent with our finding of decreased affinity of ^3H -apomorphine binding after NSP lesions. For example, it is apparent from the present data that both pre- and postsynaptic binding of apomorphine occurs. It is also probable that striatal apomorphine receptor affinity is a composite of binding to the two types of receptor. Thus elimination of a higher affinity presynaptic receptor would result in a greater relative representation of the lower affinity postsynaptic receptor and hence a higher *K*_D for apomorphine after the NSP lesions. In contrast to the apomorphine binding, haloperidol and spiperone seem to be associated primarily with

postsynaptic elements in the striatum. The present data provide no information to show to what extent, if any, the neuroleptics may bind to the presynaptic dopamine receptor.

Creese *et al.*³⁸ have shown that with receptors that have bound DA antagonists, DA agonists compete less effectively for binding than DA antagonists. Conversely, DA antagonists compete less effectively than DA agonists for receptors that have bound DA agonists. Rather than postulate the existence of two types of DA receptor, Creese *et al.*³⁸ suggested that a single receptor could exist in two forms, an agonist and an antagonist state. Our experiments cast doubt on this interpretation, as ^3H -apomorphine and ^3H -neuroleptic binding in the striatum would have been expected to show the same relative changes after the NSP lesions. However, this was not the case: in fact, striatal binding of the two ligands changed in the opposite directions. It remains possible, of course, that the lesions locked the receptors into an antagonistic state. However, this alternative would be difficult to reconcile with the known potentiation of the behavioural effects of apomorphine after NSP lesions^{27,28}. Another possibility is that there are two types of DA receptor, both located postsynaptically. In this case, the NSP lesion might change the relative numbers of each type of receptor. Again, however, an increase in ^3H -haloperidol and ^3H -spiperone receptors accompanied by a decrease in ^3H -apomorphine receptors, together with the lower affinity of apomorphine for the antagonist receptor, would be difficult to reconcile with the above behavioural observations.

It has recently been demonstrated that striatal DA-sensitive adenylate cyclase is located postsynaptically rather than on the DA terminals^{17,18}. In the SN, DA receptors on afferent terminals are coupled to adenylate cyclase, whereas DA receptors on dopaminergic neurones are not^{11–15}. This, together with the observations described here, suggests that haloperidol and spiperone bind preferentially to an adenylate cyclase-linked DA receptor. In contrast, the presynaptic DA receptor in striatum and the DA receptors on dopaminergic neurones in the SN, which preferentially bind apomorphine,

appear to be independent of adenylate cyclase. On these grounds, it is proposed that there are two types of DA receptors one of which is adenylate cyclase linked and is present on nondopaminergic neurones, and another which is autonomous of adenylate cyclase and is found on DA perikarya, dendrites and terminals. These results do not exclude the possible existence in DA neurones of adenylate cyclases that function independently of DA.

This work was supported by the MRC of Canada and the Ontario Mental Health Foundation. J.I.N. is a predoctoral fellow of the Huntington's Society of Canada. We thank Stella Atmadja for technical assistance.

J. I. NAGY

*Division of Neurological Sciences,
Department of Psychiatry, University of British Columbia*

T. LEE
P. SEEMAN

*Department of Pharmacology,
University of Toronto,
Toronto, Ontario,
Canada M5S 1A8*

H. C. FIBIGER

*Division of Neurological Sciences,
Department of Psychiatry, University of British Columbia,
Vancouver, British Columbia,
Canada V6T 1W5*

Received 8 November 1977; accepted 11 May 1978.

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Met-enkephalin inhibits *in vitro* dopamine-induced LHRH release from mediobasal hypothalamus of male rats

THE morphinomimetic pentapeptides enkephalins¹ have been identified in the central nervous system (CNS)^{2–5}. In the rat, Met-enkephalin seems to be the predominant opiate-like pentapeptide⁶; it has been found in different structures of the brain, including the anterior and the mediobasal hypothalamus (MBH)^{2–4}. Enkephalins, like morphine, have been shown to enhance the secretion of prolactin^{7–9} and growth hormone^{9,10}, and to inhibit the release of thyroid stimulating hormone¹¹, and luteinising hormone (LH) and ovulation^{12,13}. These effects can be inhibited by naloxone, an opiate antagonist; most authors have postulated that they involve a direct action at the hypothalamic level^{8–14}. In this report, we rule out a direct action of Met-enkephalin on LH releasing hormone (LHRH) release and postulate instead that Met-enkephalin modulates a dopamine-stimulated secretion.

The *in vitro* release of the neurohormone was measured by radioimmunoassay on incubated fragments from three different hypothalamic structures which contain high amounts of LHRH nerve endings¹⁵—the palisadic zone of the median eminence (rostral MBH), the infundibular sulcus (caudal MBH), and the organum vasculosum of the lamina terminalis (OVLt). We have previously demonstrated that the release of the neurohormone from this preparation can be induced by membrane depolarisation, like that resulting from exposure to high K⁺ concentrations in a Ca²⁺-dependent manner¹⁶; moreover, dopamine (DA) has been found to stimulate the release of LHRH from the palisadic zone (PZ)¹⁵, as well as, in certain conditions, from a synaptosomal fraction of the MBH¹⁷. We thus investigated the effect of Met-enkephalin on the basal and on the DA-induced release of the neurohormone from various hypothalamic fragments. The results presented here suggest that Met-enkephalin does not interfere with the basal secretion of LHRH from the median eminence, but that, instead, it

Table 1 Effects of Met-enkephalin and naloxone on *in vitro* basal LHRH release from the rostral and caudal mediobasal hypothalamus (MBH) and from the organum vasculosum of the lamina terminalis (OVLt)

	LHRH Release (pg Eq ⁻¹)		
	Rostral MBH	Caudal MBH	OVLt
Control	29.7 ± 3.0 (14)	24.0 ± 4.0 (15)	19.0 ± 3.2 (12)
Met-enkephalin 10 ⁻⁷ M	30.0 ± 4.2 (14)	21.0 ± 3.5 (12)	23.2 ± 2.7 (11)
Naloxone 5 × 10 ⁻⁶ M	29.2 ± 4.2 (12)	19.2 ± 3.5 (13)	25.0 ± 3.7 (15)
Met-enkephalin 10 ⁻⁷ M + Naloxone 5 × 10 ⁻⁶ M	30.5 ± 5.2 (14)	27.2 ± 4.5 (14)	19.5 ± 4.2 (14)

Intact male Sprague-Dawley rats (200–250 g) were killed by decapitation in the morning and tissues rapidly dissected as previously described¹⁵. Tissues were incubated individually in a shaking water bath at 37 °C in an atmosphere of 95% O₂–5% CO₂. Incubation was carried out in Locke medium (NaCl 154 (mM), KCl 5.6, CaCl₂ 2.2, MgCl₂ 1, NaHCO₃ 6, glucose 10) buffered to pH 7.2 with 2mM HEPES (Calbiochem) (incubation volume: 100 µl in 5-ml plastic tubes). Bacitracin (2 × 10⁻⁵ M Sigma) was added to prevent peptidase degradation of Met-enkephalin¹⁸ and LHRH¹⁹. At the end of the 10-min incubation period, the medium was immediately acidified with 0.1 M HCl. LHRH radioimmunoassay was carried out as previously described¹⁵. Harvey's adaptation²⁰ of factorial analysis to unequal subclass numbers was used in the statistical assessment of possible interacting effects. In the case of a significant interaction, statistical differences were examined by means of the unpaired Student's *t* test. Numbers of rats are given in parentheses.

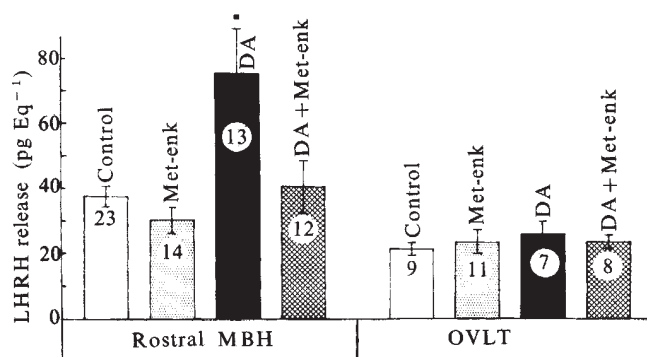


Fig. 1 Effects of dopamine (DA) and Met-enkephalin (Met-enk) on *in vitro* LHRH release from the rostral MBH and from the OVLT. DA (10^{-7} M; Calbiochem) was dissolved in Locke-HEPES medium containing ascorbic acid (5×10^{-4} M) to prevent amine degradation, Met-enk was used at a concentration of 10^{-7} M; both drugs were added at time 0. Incubation and statistical procedures are described in Table 1; ■ $P < 0.05$ versus DA + Met-enk. Numbers of animals shown in the columns.

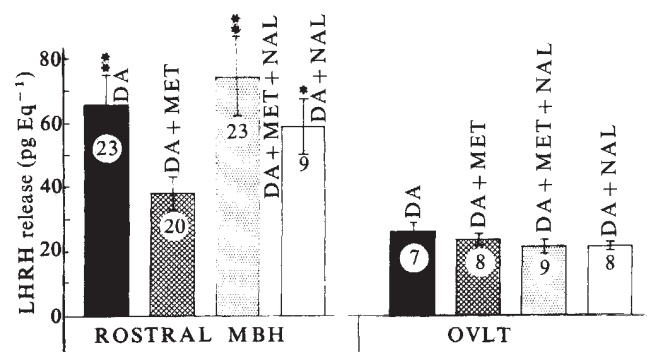
antagonises the stimulatory effect of dopamine on the release of the neurohormone.

As shown on Table 1, addition of Met-enkephalin (10^{-7} M), (a similar result was obtained with 10^{-5} M, data not shown), and/or naloxone (5×10^{-6} M) to the incubation medium does not affect LHRH release from any of the structures studied. As previously reported¹⁵, DA increases the amount of LHRH release from the PZ of the median eminence, but not from other structures (Fig. 1). This effect is abolished in the presence of Met-enkephalin (Fig. 1). In the OVLT, a structure which is rich in LHRH terminals but devoid of any significant DA innervation²¹, no effect could be obtained with DA, whether alone or in combination with Met-enkephalin.

In order to examine the specificity of Met-enkephalin action on *in vitro* DA-induced LHRH release, we tested the effect of naloxone. As shown in Fig. 2, naloxone suppresses the inhibitory effect of Met-enkephalin on DA-induced LHRH release from the PZ. However, the antagonist does not itself interfere with the stimulatory action of DA. On the OVLT, naloxone does not modify the amount of LHRH recovered from the incubation medium at the end of the incubation (Fig. 2).

Our results suggest that opiate-like peptides acting at the level of the median eminence do not effect LHRH release

Fig. 2 Effects of Met-enkephalin (Met) and naloxone (Nal) on the *in vitro* dopamine (DA)-induced LHRH release from the rostral MBH and from the OVLT. DA, Met and Nal (Endolabs) were used at concentration of 10^{-7} M, 10^{-7} M and 5×10^{-6} M respectively. See Table 1 legend for details. **, $P < 0.01$; *, $P < 0.05$ versus DA + Met. Numbers of rats given in the columns.



directly, but rather interfere with the capacity of neurosecretory nerve endings to respond to DA stimulation. This may indicate the presence of DA and opiate receptors on LHRH nerve endings of the PZ. The fact that the stimulatory action of DA on *in vitro* LHRH release could be inhibited by DA antagonists, such as pimozide and haloperidol^{15,22}, and that naloxone inhibits the action of Met-enkephalin without modifying the stimulatory effect of DA, suggests that the binding sites of DA and opiates on LHRH nerve terminals are separate.

Interactions between opiate-like peptides and dopamine have already been described²³⁻²⁵. Ferland *et al.*²⁵ postulated, on the basis of *in vivo* experiments, that the stimulation of prolactin release observed after intraventricular administration of Met-enkephalin could be partially explained by an effect on the turnover rate of DA in the median eminence. Alternatively, interactions between the opiates and the amine have also been shown to occur at a postsynaptic level^{26,27}; in the striatum, in particular, opiates block the DA-induced activation of adenylate cyclase²⁷. Although an involvement of cyclic nucleotides mediating DA effects has not yet been demonstrated in the median eminence, our results suggest that Met-enkephalin interferes with the effect of dopamine at the postsynaptic level rather than by modifying the turnover of the amine.

We thank Dr Josée Lalonde (Laval University) for statistical analysis and Miss Marie-Christine Simon for secretarial assistance. This work was supported by contracts 74/7/0804 from the Délégation Générale à la Recherche Scientifique et Technique and 1891 from the CNRS.

W. H. ROTSZTEJN
SOPHIA V. DROUVA
E. PATTOU
C. KORDON

Unité de Neuroendocrinologie (U.159),
Centre Paul Broca de l'Inserm,
2ter rue d'Alésia,
75014 Paris, France

Received 1 March; accepted 23 May 1978.

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Composition of acetylcholine receptor protein from skeletal muscle

THE isolated acetylcholine-recognition unit of the receptor protein (AChR) is characterised by its specific binding of snake venom α -neurotoxins and of nicotinic cholinergic ligands¹⁻⁴. AChR purified completely from mammalian denervated muscle consists, chiefly or entirely, of an oligomer of 41,000 molecular weight subunits. It is not clear whether this comprises the entire receptor system, or whether another component is involved^{5,6} in forming the ionic channel.

The receptor was extracted from a crude membrane preparation of denervated cat hind-leg muscles⁷, at 5°C for 2 h in 1.5% Triton X-100–50 mM phosphate buffer (pH 8), containing, as protease inhibitors, 10⁻³ M EDTA, 10⁻⁴ M phenylmethylsulphonyl fluoride–10⁻⁴ M benzethonium chloride–pepstatin (5 μ g ml⁻¹). AChR activity was assayed by measuring the binding of ³H- α -neurotoxin¹. AChR was purified by adsorption to an affinity resin, prepared⁸ by coupling α -neurotoxin (purified from venom of *Naja naja siamensis*) to CNBr-activated Sepharose 4B. After removal of unwanted protein by extensive washing with 1M NaCl in 0.2% Triton X-100–25 mM phosphate (pH 8) with similar protease inhibitors present, the AChR was eluted with 1M carbamoylcholine in the latter medium (without NaCl). Carbamoylcholine was completely removed on a DEAE–Sepharose CL-6B column¹. Specific activities at this stage (expressed as μ mol toxin binding per g protein) were in the range 3–6 μ mol g⁻¹; protein was measured with Folin–Lowry reagent, using bovine serum albumin as standard¹. Recoveries of AChR activity ranged from 20 to 50%. These values were obtained in 30 preparations each starting with 50–200 g muscle. A similar procedure was used for the purification of AChR from electric organ of *Torpedo marmorata*, to a specific activity of 10 μ mol g⁻¹.

A final stage of purification was performed with the muscle AChR by one of two methods. (1) The receptor was reappplied to the toxin affinity gel, washed and eluted as before (without the DEAE–Sepharose stage), when the specific activity was always increased: this increase was 30%–100% in five different preparations, to specific activities of 4.0–10.5 μ mol g⁻¹. In method (2), which gave a higher average yield, the AChR from the first toxin affinity elution was separated from carbamoylcholine on a Sephadex G-75 column in 75 mM Na phosphate (pH 7.4)–75 mM NaCl–1.5 mM CaCl₂–5 mM MgCl₂–0.2% Triton X-100. Since the muscle AChR is a glycoprotein^{8,9}, it was further purified by binding to a column (12 ml) of *Lens culinaris* lectin¹⁰ immobilised¹¹ on Sepharose 4B. After washing with the same medium (2 bed volumes), removing some protein but no AChR, elution was in the same medium without CaCl₂ or MgCl₂ but with 1 mM EGTA added, and with a linear gradient of 0–0.4 M α -methylglucoside and 0–0.4 M α -methylmannoside. A peak of activity was eluted, centred at 0.2 M glycosides, in over 90% yield of the bound AChR. Protein was measured as before (with the glycoside medium in the standards), and confirmed by estimation based on amino acid analysis: in two such preparations the specific activity was 9.8 and 7.5 μ mol g⁻¹—higher than that from a previous purification scheme¹ which gave a low yield; method (2) is the preferred preparative method.

The purity of the muscle AChR was verified by isoelectric focusing; as will be detailed elsewhere, one intense protein band, with isoelectric point $\sim$ pH 5.0, was visible after staining, and could be shown to contain the AChR activity. On density gradient centrifugation, at least 90% of the pure receptor, or its toxin complex¹², had $s_{20,w}$ of 9S. The purified AChR bound^{1,13} nicotinic cholinergic ligands with affinities quite similar to those obtained with membrane fragments from denervated muscle⁷; when those ligands were at their saturating concentrations, no ³H-toxin binding was detectable.

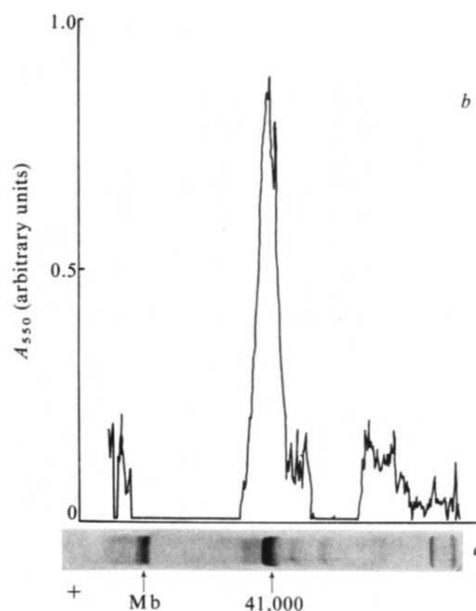


Fig. 1 Gel electrophoresis¹⁴ in SDS of AChR purified from cat denervated muscle, in 10% polyacrylamide gels (0.25 $\times$ 7.4 cm), pre-run for 1 h at 2.5 mA per tube prior to the addition of the stacking gel. The AChR solution (0.3 ml) containing 73.5 picomol of toxin-binding activity and 15.3 μ g of protein was mixed with 15 μ g of myoglobin (Mb; internal marker) and denatured in 1% SDS/1% β -mercaptoethanol–15 mM Tris-HCl, pH 6.5, at 100°C for 2 min. The standard proteins, bovine serum albumin, ovalbumin, chymotrypsinogen, myoglobin and cytochrome c, were treated similarly. All mobilities were calculated relative to myoglobin; a semilogarithmic plot was used for the determination of the unknown molecular weights. The gels were run at 5 mA per tube for 1.5 h. *a*, Gels were washed overnight in 25% isopropanol–10% acetic acid, quantitatively stained¹⁵ with 1% Fast Green (Sigma) in 7% acetic acid for 2 hours and destained in 7% acetic acid. Arrow represents MW of 41,000. *b*, Gels stained for carbohydrate by the PAS method¹⁶ and scanned at 550 nm.

The muscle AChR before the final stage of purification was analysed by SDS–polyacrylamide gel electrophoresis¹⁴, stained quantitatively¹⁵ with Fast Green, and found to contain one major protein subunit with MW 41,000 (Fig. 1*a*), with minor components present, at that stage, of MW 38,000, 50,000, 60,000 and 100,000; the proportions of these latter varied in different preparations. The 41,000 MW polypeptide also contained carbohydrate, as demonstrated by the PAS staining¹⁶ method (Fig. 1*b*). After adsorption of the intact AChR to concanavalin A–Sepharose, this 41,000 MW subunit, and the 38,000 component, were absent from a subsequent SDS-electrophoresis gel. The 38,000 MW component which contained carbohydrate was either absent or variable and very minor in the fresh preparations, and is suggested to be a proteolytic product of the 41,000 MW subunit. The 60,000 MW component seems to lack PAS-positive sugars.

Similar SDS gel electrophoresis of the AChR preparation which had been further purified to a specific activity of 9.8–10.5 μ mol per g protein using either method (1) or (2), showed that it is composed of 41,000 MW subunits, while small amounts of one or two other components are also present, which were variable in different preparations (Fig. 2). When AChR was prepared instead by a variant of method (1), using elution from the toxin column by 1% SDS instead of by carbamoylcholine, a similar pattern was obtained. Hence, the specific binding activity of muscle AChR resides either entirely in an oligomer of 41,000 MW subunits (the other material being from trace contamination), or in that oligomer with a

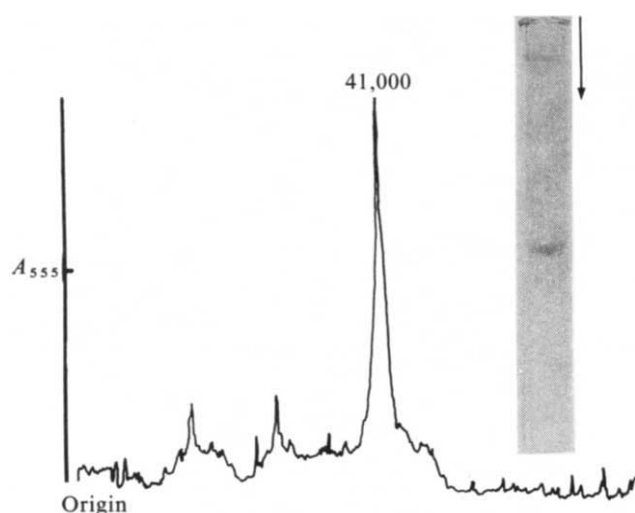


Fig. 2 SDS gel electrophoretogram and its scan, of muscle AChR purified to 9.8 μmol per g of protein using first the toxin affinity gel and then the *Lens* lectin column [method (2)]. After electrophoresis of 20 pmol AChR (performed as described for Fig. 1) the gels were washed in 25% isopropanol–10% acetic acid overnight, stained in 0.04% Coomassie G-250–3.5% perchloric acid, destained in 10% acetic acid and scanned at 555 nm. The scan ends at the right at the dye front. The MW calibration was made using a similar gel in which myoglobin was run as internal marker, and using a plot obtained from a parallel run of all of the marker proteins. The overall increase in background level of the stained gel seen above about the 40,000 MW position occurred likewise in all gels run similarly with the same volume (0.3 ml) of receptor-free sample buffer in the SDS medium and stained similarly. A major band is seen at 41,000 MW together with very much smaller amounts of two heavier components (58,000 and 68,000). Similar analysis of the AChR protein purified by method (1) to a specific activity of 10.5 μmol per g protein, again showed the major 41,000 MW band together with small amounts of heavier components and a small proportion of the 38,000 MW component.

small additional complement of heavier subunits. Actin, a potentially interfering component, was shown to be absent by separating in SDS gels an added standard of muscle actin from the muscle (or the *Torpedo*) AChR major subunit, the actin running as a sharp band of 1,500 greater MW.

Our preparation of the AChR from *Torpedo* contained, as reported by others²⁻⁴, a major subunit which in our calibrated gels migrated as having 41,000 MW; this coincided with that from muscle AChR when mixed samples were run in SDS gel electrophoresis. These and other comparisons with *Torpedo* AChR will be described elsewhere. Tritium-labelled affinity reagents for the ACh recognition site of the receptor⁴ label in *Torpedo* AChR a subunit of MW 40,000, and in *Electrophorus* AChR one of about similar size^{4,17}; these apparently correspond to the muscle AChR 41,000 subunit. In addition to this major subunit, AChR from fish electric organs has been reported by several authors to contain substantial amounts of heavier subunits^{3,4} but two other laboratories^{2,17} have found these to be insignificant, resembling our findings for muscle.

Conclusions which differ from ours have been reported by Hall and co-workers^{9,18} working with purified rat denervated leg muscle AChR. They find in SDS electrophoresis gels stained for protein, major bands at MW 45,000 and (the most prominent) 51,000, and other significant bands at 56,000, 62,000 and 110,000. *Torpedo* AChR purified in the same way yielded⁹ a major band at 44,000 MW, and minor bands at 53,000, 58,000 and 64,000. Since, as noted above, the MW of the major *Torpedo* subunit was found to be between 40,000

and 41,000 by others²⁻⁴ and by us using internally-calibrated gels, we take our muscle AChR subunit of 41,000 MW to be the same as that of Hall and co-workers at 45,000, due to differences in details of gel measurements. The presence of the larger amounts of the other subunits reported^{9,18} must be left an open question. The specific activity was stated as 7.7 and 10.9 μmol per g protein, but since only 8 μg of AChR was obtained at that level and the very low protein concentration was determined by the ³H-dansylation method¹⁹ in SDS medium using serum albumin as a standard, those values can be considerably in error, and the purity may be lower. As has been pointed out¹⁹, the dansylation of serum albumin can proceed differently to that of a test protein, especially a membrane protein, and we have observed that the AChR protein ³H-dansylates by that method to a much lower relative incorporation than does serum albumin. As a further anomaly, yet another component, of 49,000 MW, reacted with the cholinergic maleimide affinity label about equally with the 45,000 MW component in that study¹⁸, in contrast to the findings noted above with the *Torpedo* and *Electrophorus* AChR.

The highest specific activity, 10–11 μmol per g, that we have obtained with muscle AChR is the same as that reported by a number of laboratories for the purest preparations from *Torpedo* and *Electrophorus*. However, if AChR comprises a single type of polypeptide with MW 41,000 that binds toxin, a specific activity of 24 μmol per g should be obtainable, a value not approached for any reported preparation of an AChR. This discrepancy could be accounted for by (1) charge heterogeneity² in the 41,000 MW subunits, with only one type binding toxin, or (2) a partial inactivation during isolation, or (3) possibly, two identical subunits binding one toxin molecule.

Thus the protein from muscle characterised, by its binding of ligands and α -neurotoxin, as the ACh-recognition component of the receptor, has been purified to apparent homogeneity in the presence of several protease inhibitors. It contains only subunits of MW about 41,000, or those plus a low proportion of heavier material.

We thank Mr D. Green and Mr J. Skinner for technical assistance. This work was supported by the MRC.

ROBERT G. SHORR

J. OLIVER DOLLY

ERIC A. BARNARD

Department of Biochemistry,
Imperial College of Science and Technology,
London SW7, UK

Received 5 September 1977; accepted 18 May 1978.

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Ouabain-like activity in toad skin and its implications for endogenous regulation of ion transport

THE demonstration that opium alkaloids of the plant kingdom mediate their effects on animals through specific receptors¹ led to the discovery that endogenous molecules with opiate activity exist widely in animals², including man. These studies raised the possibility that other potent pharmacological agents derived from plant sources might have active analogues in higher species. Thus, cardiac glycosides are found widely in the plant kingdom³; structurally similar compounds in the animal kingdom are known to exist only in the poison glands of certain toads³. The dried skins of *Bufo* toads have been used in Chinese folk medicine for the treatment of cardiac failure⁴. Further, these poorly characterised medicinal preparations (Ch'an Su) were shown in the 1920s to contain ouabain-like compounds (bufotoxins) by chemical analysis⁵. However, although bufotoxins were shown to be a major constituent of the poison glands of *Bufo marinus*³ the possibility that similar compounds might also serve a physiological role apart from that of a defensive toxin was apparently not considered. More recently, two major observations make the consideration of such a physiological role seem reasonable. First, amphibian skin, which is a major organ for regulation of sodium and water homeostasis in these species⁶, has been shown to be rich in (Na⁺ + K⁺)-ATPase, the molecular mediator of sodium transport across the skin⁸. In fact, with toad bladder, amphibian skin has been widely used as a model of transepithelial ion transport in higher animals. Second, the pharmacological receptor for cardiac glycosides is now believed to be the (Na⁺ + K⁺)-ATPase^{9,10}, glycoside binding to this enzyme resulting in inhibition of both ATPase activity^{11,12} and associated ion transport^{13,14}. This glycoside-enzyme interaction is considered to be the basis for the cardiac stimulating activity¹⁰ for which glycosides such as ouabain and digoxin are used in the therapy of cardiac disorders. We report here that extracts of the skin of three different species of toad each contain high concentrations of a substance with all the functional properties of cardiac glycosides. The widespread existence of ouabain-like activity in an organ rich in physiologically important (Na⁺ + K⁺)-ATPase suggests a possible role for these endogenous compounds in the regulation of transepithelial ion transport.

Toads of the species *Bufo marinus* were killed by pithing, after which 2 × 2-cm segments of both ventral and dorsal skin were removed and placed on ice. Care was taken to avoid contact with the region of the poison-producing parotid glands, and no secretion of venom from these glands was observed to occur. Skin segments were weighed and then homogenised (10 cm³ per g of skin) in either 100% methanol or acid ethanol (75% ethanol, 0.2 M HCl) at 4 °C in a Sorval Omnimixer. After 14 h at 4 °C, the particulate material was removed by centrifugation, and the supernatant was evaporated to dryness in a rotary evaporator. The material was then resuspended to the original volume in aqueous buffer (140 mM NaCl, 20 mM HEPES, pH 7.4) and this solution was used in subsequent assays. After removal of skin segments, several drops of milky poison were expressed from the granular glands by pressure, and collected in plastic tubes. The glandular secretion was mixed and extracted with both methanol and acid ethanol as above. In addition, methanol extracts of the skin of two other species of toad, *Dendrophryniscus minutus* and *Atelopus ignescens* were diluted in assay buffer as above, before use.

Extracts of the skin of *Bufo marinus* inhibited ouabain binding (Fig. 1a) and inhibited ⁴²K influx into human red blood cells (Fig. 1b). In each case, the dose response of the extract paralleled that produced by ouabain (Fig. 1). Very similar degrees of inhibition were obtained with skin from ventral and dorsal regions, and extractions with methanol and acid ethanol

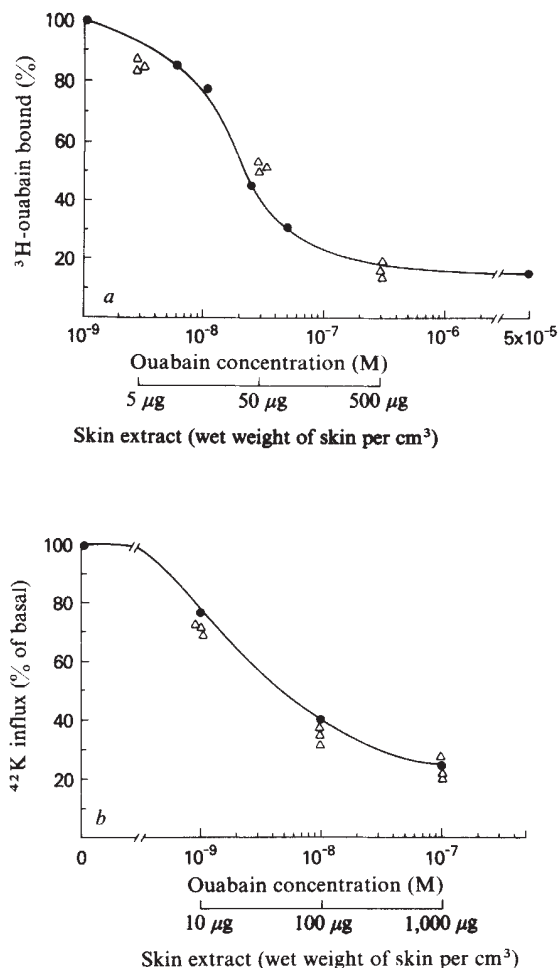


Fig. 1a Effect of ouabain and toad skin extract on binding of [³H]-ouabain to human red blood cells was assessed by the method of Gardner *et al.*¹⁵. Heparinised blood was washed four times with 140 mM choline chloride at 4 °C, the buffy coat was removed, and red cells were resuspended at a haematocrit of 15% in an assay buffer of 140 mM NaCl, 30 mM HEPES, 10 mM glucose, and 10⁻⁹ M [³H]-ouabain (8 Ci mmol⁻¹, New England Nuclear) at pH 7.4. Duplicate tubes contained ouabain or dilutions of an acid-ethanol extract of the ventral trunk skin of *Bufo marinus*, as indicated. Cells and tracer were incubated in micro-fuge tubes until steady state (60 min at 37 °C), and bound and free ouabain were separated by washing four times with 140 mM choline chloride followed by addition of 10% perchloric acid to the pellet and inversion of the tube into a scintillation vial containing Aquafuor (New England Nuclear). Data points for the extract each represent the mean of duplicate tubes in a separate experiment. Duplicates varied less than 5%. Effects of ouabain and toad skin extract on influx of K⁺ into human red blood cells. Heparinised blood was washed four times with 140 mM NaCl at 4 °C, the buffy coat was removed, the red cells were resuspended at a haematocrit of 10% in a buffer of 140 mM NaCl, and the indicated concentration of ouabain, ventral skin extract or buffer. After preincubation for one hour at 37 °C, cells were washed three times with 150 mM NaCl, 4 mM KCl, 10 mM glucose, 10 mM Tris, pH 7.4, and 10 μCi ⁴²K, 5 Ci g⁻¹. Cells were washed three times with 140 mM NaCl after an incubation period of 40 min at 37 °C. The cell pellet was treated with 10% perchloric acid, and the tubes were inverted into scintillation vials as above. Influx was determined by subtracting the ⁴²K⁺ associated with the cells at zero time from that obtained after 40 min. Influx after pretreatment with ouabain or skin extracts was expressed as a % of that after preincubation with buffer alone. Data points for the extract represent triplicate tubes in a single experiment. Influx of K⁺ seems to be more sensitive to ouabain inhibition than does [³H]-ouabain binding because cells were preincubated with ouabain and skin extracts in the K⁺ flux experiments. Δ, Skin extract; ●, ouabain.

Table 1 Effect of toad skin extract on ATPase activity

Extract (wet weight per cm ³)	ATPase activity (% of control)
10 µg	76.0
100 µg	67.5
1 mg	43.9
2 mg	23.2

Purified ATPase from the electric eel was prepared as previously described¹⁶. ATPase assay was carried out in a total volume of 100 µl in a buffer containing 25 mM KCl, 100 mM NaCl, 5 mM ATP, 5 mM MgCl₂, 40 mM Tris, pH 7.4, 2 µCi [γ -³²P]ATP, 100 Ci mmol⁻¹, and 20 µg eel ATPase preparation, in the presence and absence of skin extract. After incubation for 30 min at 24 °C, reaction was stopped by the addition of 200 µl of 1% ammonium molybdate in 2 M H₂SO₄ at 4 °C. The sample was vortexed, 1.0 cm³ of isobutanol was added, and the sample was vortexed again. After centrifugation at 3,000 r.p.m. for 5 min at 4 °C, a 700-µl aliquot of the upper phase was added to 15 cm³ of ACS scintillation liquid and counted in a scintillation counter. Data are expressed as ATPase activity in the presence of extract/ATPase activity in the absence of extract $\times 100$, and each number represents the average of two experiments which varied by less than 5%. The activity of the enzyme was 2 µmol of P per mg per min.

produced similar results. Further, the *Bufo marinus* skin extract was a potent inhibitor of purified (Na⁺ + K⁺)-ATPase from the electric organ of *Electrophorus electricus* (Table 1). Based on the binding competition and ⁴²K flux studies, an amount of extract equivalent to 30–100 µg wet weight of skin had a potency equal to approximately 10⁻⁸ M ouabain. If the active substance(s) in toad skin have a biological potency equal to that of ouabain and a similar molecular weight (that of ouabain is 584), then approximately 0.01% of the wet weight of the toad skin would be composed of ouabain-like components.

Skin extracts of *Dendrophryniscus minutis* and *Atelopus ignescens*, when tested in the ³H-ouabain binding competition assay, had concentrations of extractable ouabain-like activity indistinguishable from that seen in skin of *Bufo marinus*. These toads were selected because they have no large poison glands. In addition, the pure venom of *Bufo marinus* contained only 50 times more activity by weight than did the skin of each species of toad. Finally, the specificity of ouabain-like activity of skin was demonstrated by the fact that an identically prepared extract of toad muscle had no inhibitory activity when tested over the same range of dilutions.

The chemical nature of the 'endogenous ouabain-like activity' of toad skin has not yet been investigated in detail. The activity is dialysable, and withstands boiling for 15 min. It is not destroyed by treatment with 1 mg ml⁻¹ trypsin for one hour. These data, and the close similarity of the actions of ouabain and the endogenous compounds in three separate assays, suggest that some, if not all, of the activity which we have observed was caused by endogenous bufotoxins. However, the possibility remains that the activity is caused by molecular species chemically distinct from traditional bufotoxins.

With the finding of high concentration of ouabain-like activity in toad skins, it will be necessary to re-evaluate previous studies of the sodium transport and ATPase activities of this organ. Perhaps the observation that high concentrations of ouabain are needed to inhibit the (Na⁺ + K⁺)-ATPase in the skin (and bladder) of *Bufo marinus* is attributable to endogenous ouabain present in these tissues. In this regard, purification of this (as well as other ATPase preparations) has been noted to be associated with a greater than expected increase in enzyme activity, also consistent with the presence of endogenous inhibitors. The function of endogenous ouabain-like activity in the skin of these toads remains to be elucidated. The ouabain-like bufotoxins found in the large poison glands of *Bufo* toads have a role in the self-defence of these species.

However, a defensive role for these ouabain-like molecules might be a specialised adaptation from a pre-existing regulatory function. It is possible that the toads which we have studied, even the two species with no known poison glands, have smaller granular glands distributed throughout the skin, and that these small glands form, store and release ouabain-like compounds as a poison for solely defensive purposes. However, the fact that ouabain specifically inhibits (Na⁺ + K⁺)-ATPase, an enzyme which subserves a critical physiological function of toad skin, makes this possibility less appealing and suggests to us that the endogenous ouabain-like activity in toad skin might have a regulatory role in the control of the ATPase in these species.

Further, as the ouabain binding site on the (Na⁺ + K⁺)-ATPase has been rigorously conserved through evolution, it may be speculated that endogenous substances might regulate (Na⁺ + K⁺)-ATPase of higher species by binding to this site. Such regulators, if they existed, would have important consequences for our understanding of the regulation of transmembrane ion transport and energy utilisation.

I thank Drs Jesse Roth and C. Ronald Kahn for their support and criticism during these studies, Dr J. Daly for skin extracts and helpful discussions, Dr R. W. Albers for the ATPase preparation and suggestions concerning the ATPase assay, Drs J. Gardner, G. Stewler, L. C. Harrison and C. Grunfeld for helpful discussions, P. Nakada and A. Itin for assistance, and C. Shinn for aid in preparation of the manuscript.

Note added in proof: Since submission of this manuscript I have become aware of a report of isolation of molecules chemically related to bufotoxins from the skin of the Japanese toad¹⁷.

JEFFREY S. FLIER

Diabetes Branch, National Institutes of Arthritis, Metabolism, and Digestive Diseases, National Institute of Health, Bethesda, Maryland 20014

Received 25 January; accepted 24 April 1978.

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Disorganised and 'excessive' reinnervation of frog cardiac ganglia

THE vertebrate nervous system is characterised by the existence of specific patterns of interconnections established during embryonic development. When nerve pathways are disrupted, axonal regeneration re-establishes the original patterns in some cases^{1–3} but not in others^{4–6}. Little is understood about how the specific patterns develop and why they are, or are not, reproduced after injury. Where it has been studied, it seems that target cells are initially innervated by a greater number of axons than in the fully developed tissue and that the 'excessive' synaptic inputs disappear with maturation^{7–9}. Regeneration of motor axons after damage to muscle innervation recapitulates

Table 1 Innervation pattern of neurones on the left branch of control and reinnervated cardiac ganglia in the frog

	Animals	No. of Neurones	% Of cells innervated by		
			Left vagus	Right vagus	Both vagi
Control ganglia	15	373	87.6 ± 3.3%*	51.9 ± 6.2%†	39.9 ± 5.4%†
Reinnervated ganglia	18	500	82.6 ± 3.0*	81.3 ± 3.8†	66.9 ± 3.4†

Data for reinnervated ganglia are from frogs 29–154 d after both cardiac branches of the vagus nerves had been crushed near the heart, at a time when innervation was nearly complete (see Fig. 2). Values in columns 3–5 represent the mean % ($\pm$ s.e.m.) of neurones innervated by the left, the right and by both vagus nerves, respectively. After reinnervation, there was no significant difference in the % of cells innervated by the left and right vagus nerves. In this and in Table 2, statistical significance was determined by the Wilcoxon rank sum test.

* Not significant.

† $P < 0.01$.

this developmental process¹⁰, although the original pattern of specific connections may not be restored. Comparable information about reinnervation in nerve–nerve circuits is not known. For example, are more synaptic connections than normal also established during regeneration after damage to presynaptic axons? And if so, is the specificity of the connections thereby altered? We report here our studies on the parasympathetic cardiac ganglion of adult frogs which show that neurones do receive more synaptic inputs during regeneration, and that this excess is associated with an apparent failure to restore the original innervation pattern.

The cardiac ganglion of the frog, which lies in the interatrial septum, is well suited for studying these questions. Individual cells and preganglionic nerve terminals can be seen in the living isolated preparation¹¹. Furthermore, neurones receive synapses only from the two cardiac branches of the vagus nerves, which can be drawn into separate suction electrodes and stimulated independently. We impaled ganglion cells with high-resistance microelectrodes and recorded synaptic responses evoked by stimulating the left and right branches of the vagus nerves, as previously described¹². The number of presynaptic axons innervating each neurone was determined by gradually increasing the stimulus intensity to each vagus nerve in turn and counting the number of increments in the amplitude of the evoked excitatory postsynaptic potentials^{7–9}. This procedure gives us the minimum number of presynaptic inputs. Commonly, only one or two inputs were observed, although sometimes as many as six could be distinguished (see below).

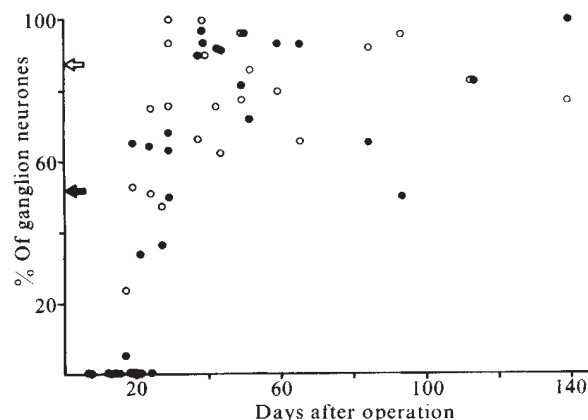
To study regeneration, we carried out the following surgical procedures. Vagus nerves to the heart were exposed near the sinus venosus by splitting and retracting the sternum in anaesthetised frogs (submerged in ice-cold 10% alcohol before the operation and kept on ice during the surgery). Both cardiac branches of the vagus nerves were crushed with fine forceps less than 3 mm from the heart. The sternum and overlying skin were sutured closed and the animals allowed to recover at room temperature. At various survival times after the operation, the interatrial septum was dissected from the heart and the innervation of the ganglion cells mapped (see above).

In the frog, cardiac ganglion cells lie along right and left branches of the vagus nerves in the interatrial septum. To simplify these studies, we have examined cells on the left branch only. Normally, the majority (88%) of the neurones on this branch of the ganglion are innervated by the left nerve and 52% receive synaptic inputs from the right vagus nerve through a chiasm¹². Therefore, about 40% of the cells are innervated by both right and left vagus nerves (Table 1). In operated animals, regenerating preganglionic axons began to re-establish connections with neurones about 17 d after the vagus nerves had been crushed. Within four weeks, almost every ganglion cell was reinnervated. The time course of reinnervation is shown in Fig. 1. As shown by open circles, the left vagus reinnervated the same percentage of neurones as in control animals. Axons from the right vagus nerve reinnervated these cells at about the same rate (solid circles in Fig. 1). Moreover, the right vagal axons re-established connections with about the

same percentage of neurones as did the regenerating left axons (Table 1). Thus, the right nerve clearly innervated a greater percentage of neurones after regeneration than in normal ganglia. It follows that the percentage of neurones which received innervation by both vagus nerves increased (from 40% in control animals to 67% in operated animals). These results show that there was no preference between right and left regenerating preganglionic axons and that the original innervation pattern was not restored.

Not only did regeneration fail to restore the normal relative left and right preganglionic innervation patterns, but in addition there were more axons innervating each cell than normally. Figure 2 shows that in control animals, about equal proportions of neurones received one and two preganglionic fibres. Only about 13% of cells were innervated by more than two axons. After reinnervation, the percentage of cells receiving more than two axons increased to 38% and many ganglion cells received four or more inputs. The average number of inputs in control animals was 1.7 and in operated animals was 2.3 (Table 2). This result has also been shown by Dennis and Sargent¹³. On detailed analysis, we found that the excess inputs

Fig. 1 Time course of reinnervation of neurones on the left branch of cardiac ganglia after both vagus nerves had been crushed near the heart. The abscissa is days after the operation and the ordinate is % of cells receiving responses evoked by stimulating the left (○) and the right (●) vagus nerves. Each symbol represents data from one ganglion (18–33 neurones tested). The arrows on the ordinate are the mean % of neurones innervated by the left (⇐) and right (⇒) vagus nerves in control animals (see Table 1). The rate of reinnervation is about equal for right and left vagus nerves and the % of cells reinnervated by either vagus is similar, in contrast with the innervation pattern in normal animals.



arose chiefly from the right vagus nerve. The number of inputs per cell from the right vagus increased about twofold after reinnervation, with no significant change in the number of left inputs (Table 2).

The increase in multiple innervation was not caused by the development of intrinsic postganglionic axon collateral synapses which can be observed after prolonged loss of pre-ganglionic fibres (ref. 14 and C.-P.K. and S.R., unpublished

Table 2 Average number of synaptic inputs per cell for neurones in the cardiac ganglia of frogs

	Left vagal inputs	Right vagal inputs	Total inputs
Control ganglia	1.15 ± 0.07*	0.59 ± 0.08†	1.73 ± 0.07†
Reinnervated ganglia	1.16 ± 0.08*	1.10 ± 0.08†	2.27 ± 0.11†

Data are taken from the same control and reinnervated ganglia shown in Table 1. Each column shows the average ($\pm$ s.e.m.) of the ratio of left, right and total vagal inputs divided by the number of ganglion cells impaled in a frog. After reinnervation, the main change in innervation was a 2-fold increase in the number of inputs per cell from the right vagus nerve. There was no significant increase in left vagal inputs after reinnervation.

* Not significant.

† $P < 0.01$.

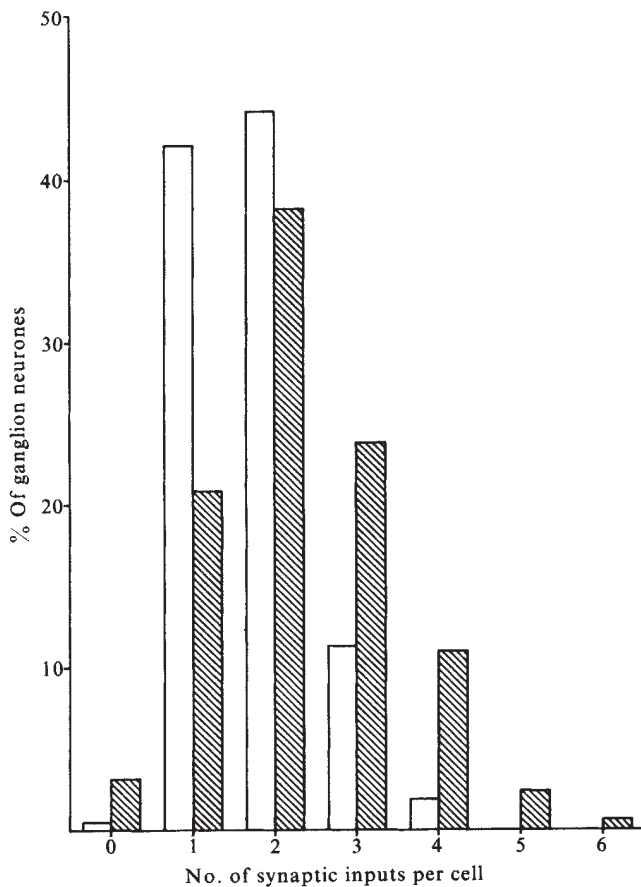


Fig. 2 Histograms of multiple innervation of parasympathetic neurones in normal and reinnervated cardiac ganglia. The abscissa is the number of preganglionic inputs per cell, determined by recording intracellular responses evoked by stimulating both branches of the vagus nerves (see text). The ordinate is % of ganglion cells. In control ganglia (open bars) the average number of inputs per cell (15 frogs, 373 neurones) was 1.7, and in animals 29–154 d after both cardiac branches of the vagus nerves had been crushed near the heart (hatched bars), the average number of inputs per cell (18 frogs, 500 neurones) was 2.3 (see Table 2).

observations). In three frogs in which we tested for this possibility 41–66 d after the operation (at a time when regeneration was complete and increased multiple innervation clearly established, see Fig. 1) there were no intrinsic synaptic connections. Similarly, electrical coupling between ganglion neurones¹⁵, which might also contribute to an apparent excess in synaptic inputs, was not observed either.

These results show that after damage to the innervation of the cardiac ganglion, preganglionic axons rapidly regenerate but do not restore the original pattern of connections. Significant differences exist. First, regeneration does not

restore the original organisation of left relative to right innervation; both vagus nerves have an equal tendency to reinnervate neurones. Second, ganglion cells are reinnervated by more preganglionic axons than normal. This increase is due to an excess number of preganglionic terminals from the contralateral vagus nerve (which is also the basis for the above failure to restore the original innervation pattern).

The lack of restoration of the original pattern of innervation may represent what is commonly referred to as 'nonselective' reinnervation. That is, although we do not know the detailed mechanisms whereby growing nerve terminals synapse with target cells, it may be important whether a cardiac ganglion cell fails to 'select' a left or a right vagal terminal. In dogs, the influence of some small vagal cardiac branches differs from that of others¹⁶, suggesting that the precise pattern of reinnervation after damage may be important, but this is not known for the frog. In any case, after damaging both vagal inputs, we found no evidence for any such 'selectivity' and it seemed that neurones would accept either left or right preganglionic inputs about equally. Experiments are in progress to determine whether this increased multiple innervation will be eliminated and the original innervation pattern restored at longer survival times.

We thank Drs W. Betz, J. Caldwell, S. Frenk, W. Proctor and R. Ribchester for their assistance in preparing our manuscript. This project was supported in part with the help of the Colorado Heart Association, NIH, and the Edward Mallinckrodt, Jr Foundation.

CHIEN-PING KO

STEPHEN ROPER

Departments of Anatomy and Physiology,
University of Colorado Medical Center,
4200 East Ninth Avenue,
Denver, Colorado 80262

Received 27 January; accepted 24 May 1978.

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Pharmacological evidence for 'fast' sodium channels in nonspiking neurones

It is becoming increasingly clear that not all neurones encode information by frequency modulation of trains of action potentials. Several sensory cells and interneurons have been identified in both vertebrates and invertebrates that transmit in an entirely nonimpulsive (or 'nonspiking') manner¹⁻⁸. The neuropharmacology of nonimpulsive neurones can be conveniently studied on the muscle receptor of the basal joint of the crab walking leg¹⁻³. Receptor potentials recorded intracellularly from the afferent neurones of this system are sodium dependent but essentially unaffected by tetrodotoxin (TTX)³. As we report here, however, veratridine mediates a sodium dependent increase in membrane conductance in these neurones that is potentially blocked by TTX. Thus, exposure to veratridine evidently sensitises the nonimpulsive membrane to the effects of TTX.

TTX is well recognised for its ability to block sodium-dependent action potentials, although it leaves nonimpulsive

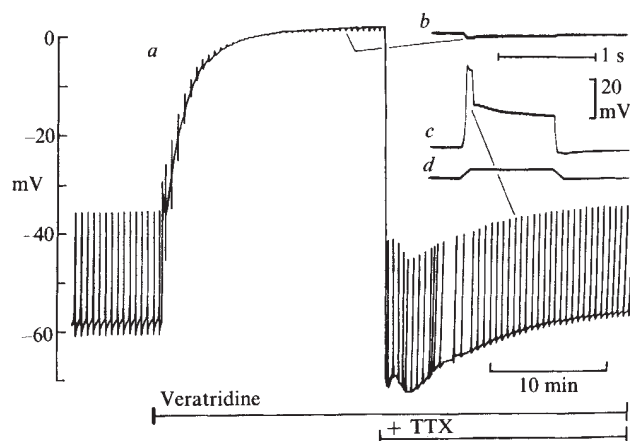


Fig. 1 *a*, Chart recording of T fibre membrane potential with superimposed receptor potentials, showing effects of veratridine and TTX. Inset shows simultaneous oscilloscope traces of representative receptor potentials (indicated on *a*), recorded *b*, during the plateau of the veratridine response and *c*, after subsequent addition of TTX; the ramp stimulus waveform is also displayed (*d*: stretch upwards). The isolated receptor organ together with attached ganglion was pinned in crab saline to the Sylgard floor of a special chamber, and its distal end connected to a servo device for controlled stretching and tension monitoring. Resting length of the receptor was generally set to about 9 ± 1 mm, this being slightly below the mid-resting length *in situ*. Conventional glass microelectrodes (20–50 M Ω) were inserted into the S and T fibres to record membrane potentials. Receptor potentials were elicited every 30 s by 0.5-mm amplitude ramp stretches of the receptor muscle. Solutions were changed using a multi-way tap, with constant flow rates of between 5 and 10 ml min⁻¹, the bath volume being about 3 ml. Normal medium contained: Na 500 mM, K 12 mM, Ca 12 mM, Mg 20 mM, Cl 576 mM, buffered to pH 7.3 with 2 mM TES. Addition of 50 μ M veratridine caused a rapid depolarisation, the membrane potential becoming eventually positive. The receptor potential amplitude decreased to zero, becoming negative-going at positive membrane potentials. TTX 1 μ M caused an immediate rapid repolarisation to pre-veratridine levels. The receptor potential amplitude also returned to normal in the presence of TTX.

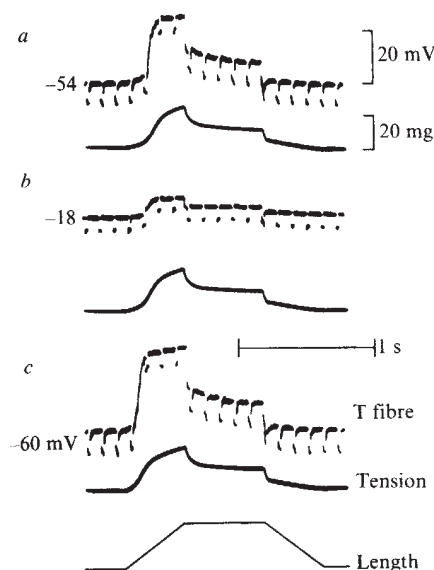


Fig. 2 Oscilloscope records showing effects of veratridine and TTX on T fibre membrane conductance. Brief constant current (10 nA) pulses were injected at regular intervals during receptor muscle stretch and rest through a second microelectrode impaling the fibre. *a*, Upper trace: the receptor potential with superimposed current pulses in normal medium. The membrane conductance increased (decreased in pulse amplitude) during the receptor potentials. Lower trace: tension response of receptor muscle to applied stretch. *b*, Traces as in *a* but with 50 μ M veratridine in the bathing medium. Membrane conductance was increased, whereas tension response remained the same. *c*, As in *a* but in presence of 50 μ M veratridine and 1 μ M TTX. Membrane conductance returned to normal on addition of TTX. Bottom trace shows the ramp stimulus waveform (stretch upwards).

sodium-dependent electrical activity unaffected⁹. Its action seems to be confined to voltage-dependent sodium channels, that is, the so-called fast sodium channels. Veratridine is used increasingly in neurobiology to open sodium channels. When applied to axons this drug produces repetitive action potentials of long duration, and at higher concentrations large sodium-dependent depolarisations¹⁰. As both these effects are blocked by TTX, veratridine seems also to act on the fast sodium channel. Similar results in the crayfish abdominal stretch receptor, without any concomitant effect of veratridine on the stretch-evoked generator (that is, 'receptor') potentials, were interpreted as indicating a specific action of veratridine on the sodium channels of the regenerative membrane of this sensory neurone and not on its receptive (transducer) membrane¹¹.

For our experiments we used the thoracic-coxal muscle receptor organ isolated from the posterior pereopod segment of the crab *Carcinus maenas* (reviewed in ref. 1). Each receptor is innervated by two large sensory dendrites (S and T fibres) with cell bodies which lie within the thoracic ganglion. Sodium-dependent receptor potentials are produced in the fine dendritic terminations by stretch of the receptor, and propagate non-impulsively and decrementally along the sensory fibres to the thoracic ganglion.

In the present study, inclusion of veratridine in the medium superfusing the receptor elicited large depolarisations in both S and T fibres (Fig. 1). In many cases this effect was so large that the membrane potential became positive. The receptor potentials decreased progressively in amplitude as the depolarisation proceeded, and at about zero or slightly positive membrane potentials they reversed in polarity to negative-going. These

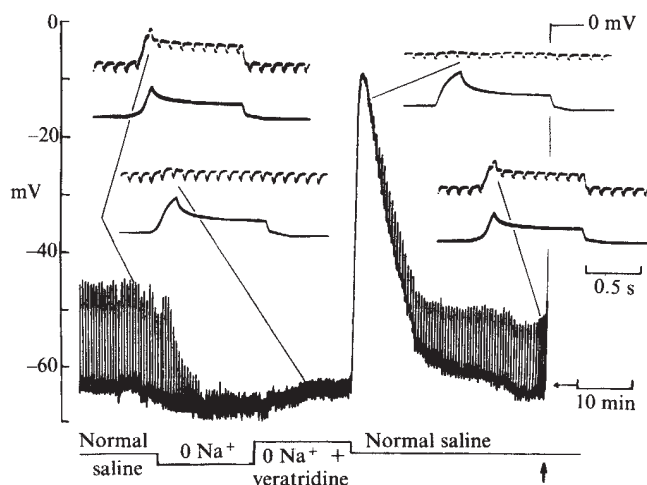


Fig. 3 Delayed effects of veratridine in sodium-free saline on T fibre membrane conductance and potentials. The chart recording is shown together with sample oscilloscope traces of selected receptor potentials and underlying tension responses (to the ramp stretches—not shown). Arrow indicates electrode withdrawal at the end of recording. Removal of sodium caused slight hyperpolarisation and progressive reduction of receptor potential amplitudes. In sodium-free medium, 50 μ M veratridine was ineffective in producing either depolarisation or an increase in membrane conductance. On return to normal medium, however, a massive transient depolarisation was observed, during which the membrane conductance increased. Both parameters returned slowly to normal. Similar large depolarisations, on return to normal medium following veratridine treatment, occurred even with long intervening periods in sodium-free medium without veratridine, indicating that the effect of veratridine is persistent.

effects of veratridine were only slowly reversible on return to normal medium, complete recovery of the membrane potential not always being observed. In contrast, a very rapid repolarisation was seen on addition of TTX to the chamber during exposure to veratridine (Fig. 1). In the initial phase of this effect the membrane potential usually exceeded that recorded before veratridine treatment, but then returned to normal in the continued presence of both TTX and veratridine. Wash-out of the drugs produced a slight decrease in membrane potential which subsequently recovered to pre-veratridine levels.

The conductance of the membrane, as monitored by injection of brief constant current pulses (10 nA) through a second microelectrode in the fibre, was increased by veratridine but returned to normal when TTX was added (Fig. 2). When sodium was removed from the perfusing saline (choline replacement), the membrane potential hyperpolarised slightly (5–10 mV). Subsequent exposure to veratridine produced neither depolarisation nor an increase in membrane conductance (Fig. 3). Return of sodium resulted in a rapid depolarisation and a corresponding increase in membrane conductance (Fig. 3). This effect was presumably due to the passage of sodium through channels previously opened by veratridine. Similarly, removal of sodium from the medium surrounding S and T fibres that had been depolarised in veratridine containing normal saline caused a rapid repolarisation despite the continued presence of the drug. Re-admission of sodium elicited a rapid TTX-sensitive depolarisation.

These results demonstrate that nonimpulsive neurones are depolarised by veratridine in a way directly comparable to impulse propagating neurones. Veratridine-mediated depolarisation in both cell types is sodium dependent, and due to an increase in sodium permeability of the membrane that is blocked by TTX. Thus the action of veratridine is not confined to neurones conducting sodium-dependent action potentials. In fact veratridine also affects several types of non-neuronal cells

the normal physiologically evoked electrical responses of which do not involve 'fast' sodium channels. The best example of this is the pancreatic β cell^{12,13}, and recent observations show that smooth muscle cells (D.A.L., unpublished) and pancreatic acinar cells (E. K. Matthews, personal communication) also respond to veratridine.

Do we have therefore to abandon the hypothesis that the action of veratridine is confined to the fast sodium channel? A variety of studies involving kinetic¹⁴, electrophysiological^{15,16}, and TTX binding techniques¹⁷, have consistently demonstrated that veratridine and TTX interact at the same receptor, albeit at different sites within the complex. In addition, voltage clamp studies on the node of Ranvier indicate that veratridine modifies existing sodium channels rather than creating new ones¹⁸.

The most likely explanation of our results, therefore, is that nonimpulsive neuronal membranes contain a macromolecular complex similar to that constituting the fast Na channel of impulsive membranes, that the appropriate components of the channel complex are present to enable activation and block by veratridine and TTX, respectively; but that the complex is either insufficiently differentiated, lacking the appropriate voltage-dependent gating mechanisms, or in some way suppressed so that sodium spike generation does not occur. It seems that this type of veratridine-sensitive channel is present in many excitable cells, even in those the normal activity of which is unaffected by TTX. One possibility is that some active processes (or principle) exist that tend to maintain the otherwise viable channel in the closed configuration, so that the membrane is in a permanent state of refractoriness. Recent evidence from other nonspiking neurones—those in the blowfly visual neuropil¹⁹—shows that spike generation can occur in response to physiological stimulation when the cell is steadily hyperpolarised by current injection, suggesting that charge distribution across the membrane is one factor determining the normal equilibrium between closed and open states of the sodium channel. However, the exact mechanism responsible for suppression of pharmacologically demonstrable sodium channels in nonimpulsive neurones remains to be elucidated.

D. A. LOWE

Department of Pharmacology,
Sandoz Ltd, Basle, Switzerland

B. M. H. BUSH

Department of Physiology,
University of Bristol, Bristol, UK

S. H. RIPLEY

Department of Physiology,
University of Natal,
Durban, South Africa

Received 30 March; accepted 1 June 1978.

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Amino acid sequence homology between polyoma and SV40 tumour antigens deduced from nucleotide sequences

POLYOMA and SV40 are DNA tumour viruses with very similar structures, genetic organisations and mechanisms of transformation¹. The late regions of the genome of both viruses code for three structural proteins of the viral capsid; the early regions code for at least two forms of tumour antigen, one with an approximate molecular weight of 100,000 (large-T antigen) and one with an approximate molecular weight of 20,000 (small-t antigen)²⁻⁵. The only region of DNA homology between the two viruses, as detected by nucleic acid hybridisation techniques, is a small segment in the late region at the

position of the overlap of the genes for VP2 and VP3 with VP1⁶⁻⁷. The nucleotide sequence of the entire SV40 genome has been determined^{8,9}, and the sequence of polyoma is now becoming available. A comparison of the nucleotide sequence near the origins of DNA replication of the two viruses has indicated homologies in nucleotide sequences previously undetected by hybridisation¹⁰. We present evidence here for nucleotide homologies in regions of the viral genomes that code for portions of t antigen and the T antigen.

The polyoma and SV40 nucleotide sequences shown in Fig. 1 represent continuous stretches of DNA having the same polarity as the early messenger RNA in the 5' to 3' orientation. The sequences start, in each case, with the first potential initiator triplet to the 3' side of the origin of replication. The polyoma nucleotide sequence represents a portion of the sequence, being presented elsewhere, of polyoma DNA from near the *Hpa*II 3/5 junction to the *Hpa*II 4/*Hae*III 18 junction¹⁸. The first potential polyoma initiator triplet is at position 188, and the polyoma nucleotide sequence given in Fig. 1 begins at that position. From there to the *Hpa*II 4/*Hae*III 18 junction at

Fig. 1 Nucleotide and deduced amino acid sequences of portions of the early regions of polyoma (Py) and SV40. The sequences have the same polarity as the early mRNA in the 5' to 3' direction, and start with the first potential initiator triplet after the origin of DNA replication. The sequences of polyoma have been determined on both strands of the DNA, but only the L strand sequences are presented. All polyoma nucleotide sequence analysis was done by the method of Maxam and Gilbert¹⁶, on fragments of viral DNA produced by restriction enzyme cleavages described elsewhere¹⁷. Labelling of the fragments at both 5' and 3' ends and details of the sequencing methods are being presented in a separate communication¹⁸. The SV40 sequence has been determined by Reddy *et al.*⁸ and Fiers *et al.* The polyoma sequence is incomplete, and we have not yet identified the termination triplet at its 3' end.

Py	188	Met	Asp	Arg	Val	Leu	Ser	Arg	Ala	Asp	Lys	Glu	Arg	Leu	Leu	Glu	Leu	Lys	Leu	Pro
SV40		ATG	GAT	AGA	GTT	CTG	AGC	AGA	GCT	GAC	AAA	GAA	AGG	CTG	CTA	GAA	CTT	CTT	CTT	CCC
		Met	Asp	Lys	Val	Leu	Asn	Arg	Glu	Glu	Ser	Leu	Gln	Leu	Met	Asp	Leu	Gly	Glu	
Py	248	Arg	Gln	Leu	Trp	Gly	Asp	Phe	Gly	Arg	Met	Gln	Gln	Ala	Tyr	Lys	Gln	Gln	Ser	Leu
SV40		AGA	CAA	CTA	TGG	GGG	GAT	TTT	GGA	AGA	ATG	CAG	CAG	GCA	TAT	AAG	CAG	CAG	TCA	CTG
		Arg	Ser	Ala	Trp	Gly	Asn	Ile	Pro	Leu	Met	Arg	Arg	Ala	Tyr	Lys	Lys	Lys	Cys	CTA
Py	308	Leu	His	Pro	Asp	Lys	Gly	Gly	Ser	His	Ala	Leu	Met	Gln	Glu	Leu	Asn	Ser	Leu	Trp
SV40		CTG	CAC	CCA	GAC	AAA	GGT	GGA	AGC	CAT	GCC	TTA	ATG	AAG	GAA	TTG	AAC	AGT	CTC	GGA
		Leu	His	Pro	Asp	Lys	Gly	Gly	Asp	Glu	Glu	Lys	Met	Lys	Lys	Met	Asn	Thr	Leu	Lys
Py	368	Phe	Lys	Thr	Glu	Val	Tyr	Asn	Leu	Arg	Met	Asn	Leu	Gly	Gly	Thr	Gly	Phe	Gln	Val
SV40		ACA	AAA	ACT	GAA	GTA	TAC	AAT	CTG	AGA	ATG	AAT	CTA	GGA	GGG	ACC	GGC	TTC	CAG	GTA
		Lys	Met	Asp	Gly	Val	Lys	Tyr	Ala	His	Gln	Pro	Asp	Phe	Gly	Gly	Phe	Trp	Asp	Ala
Py	428	Arg	Leu	His	Ala	Asp	Gly	Trp	Asn	Leu	Ser	Thr	Lys	Asp	Thr	Phe	Gly	Asp	Arg	Tyr
SV40		AGA	CTA	CAT	GCG	GAT	GGG	TGG	AAT	CTA	AGT	ACC	AAA	GAC	ACC	TTT	GGT	GAT	AGA	TAC
		Arg	Leu	His	Ala	Asp	Gly	Trp	Asn	Leu	Ser	Thr	Lys	Asp	Thr	Phe	Gly	Asp	Arg	Tyr
Py	488	Tyr	Gln	Arg	Phe	Cys	Arg	Met	Pro	Leu	Thr	Cys	Leu	Val	Asn	Val	Lys	Tyr	Ser	Ser
SV40		TAC	CAG	CGG	TTC	TGC	AGA	ATG	CCT	CTT	ACC	TGC	CTA	GTA	AAT	GTT	AAA	TAC	AGC	TCA
		Tyr	Gln	Arg	Phe	Cys	Arg	Met	Pro	Leu	Thr	Cys	Leu	Val	Asn	Val	Lys	Tyr	Ser	Ser
Py	548	Ser	Cys	Ile	Leu	Cys	Leu	Leu	Arg	Lys	Gln	His	Arg	Glu	Leu	Lys	Asp	Lys	Cys	Asp
SV40		AGT	TGT	ATA	TTA	TGC	CTG	CTT	AGG	ATG	CAA	CAT	AGA	GAG	CTC	AAA	GAC	AAA	TGT	GCC
		Ser	Cys	Ile	Leu	Cys	Leu	Leu	Arg	Lys	Gln	His	Arg	Glu	Leu	Lys	Asp	Lys	Cys	Asp
Py	608	Arg	Cys	Leu	Val	Leu	Gly	Glu	Cys	Phe	Cys	Leu	Glu	Cys	Tyr	Met	Gln	Trp	Phe	Gly
SV40		AGG	TGC	CTA	GTA	CTT	GGA	GAA	TGT	TTT	TGT	CTT	GAA	TGT	TAC	ATG	CAA	TGG	TTT	GGA
		Arg	Cys	Leu	Val	Leu	Gly	Glu	Cys	Phe	Cys	Leu	Glu	Cys	Tyr	Met	Gln	Trp	Phe	Gly
Py	668	Pro	Thr	Arg	Cys	Val	Leu	Asn	Leu	Tyr	Ala	Asp	Phe	Ile	Ala	Ser	Met	Pro	Ile	Asp
SV40		CCA	ACC	CGA	GAT	GTG	CTG	AAC	CTG	TAT	GCA	GAC	TTC	ATT	GCA	AGC	ATG	CCT	ATA	TGG
		Pro	Thr	Arg	Cys	Val	Leu	Asn	Leu	Tyr	Ala	Asp	Phe	Ile	Ala	Ser	Met	Pro	Ile	Asp
Py	728	Leu	Asp	Leu	Asp	Val	His	Ser	Tyr	Val	Asn	Pro	Ser	Lys	Tyr	Gln	Glu	Gly	Tyr	Trp
SV40		CTG	GAC	CTG	GAT	GTG	CAC	AGC	GTG	TAT	AAT	CCA	AGT	AAG	TAT	CAA	GAG	GGC	GGG	TGG
		Leu	Asp	Leu	Asp	Val	His	Ser	Tyr	Val	Asn	Pro	Ser	Lys	Tyr	Gln	Glu	Gly	Tyr	Trp
Py	788	Phe	Thr	Ala																
SV40		TTT	ACG	GCC																

position 796, there is only one continuous 'open' reading frame; the other two possible frames are 'closed' by multiple termination codons. The potential translation product of the open frame is presented, together with the nucleotide sequence. The SV40 sequence, shown in Fig. 1, is as determined by Fiers *et al.* It begins with the initiator triplet at position 80 and ends with the first termination triplet at position 602 according to the nomenclature of Fiers *et al.*⁹. The corresponding position of the initiation triplet as determined by Weissman *et al.* is 5101 (ref. 8). This sequence, in a single open reading frame, can code for a polypeptide 174 amino acids long. Identical bases are indicated by connecting lines, and identical amino acids by boxes. The nucleotide homologies are maximised by assuming deletions in three portions of SV40, corresponding to positions 476–490, 533–535 and 605–607 of the polyoma sequence. The first termination triplet in the SV40 nucleotide sequence is located at position 602, corresponding to position 740 of the polyoma virus DNA. As shown in Fig. 1, 522 SV40 and polyoma virus base pairs can be aligned to give identical positions for 223 base pairs. The homology is particularly pronounced in two regions corresponding to positions 188–391 and 545–664 on the polyoma DNA.

The deduced amino acid sequences for both polyoma and SV40 tumour antigens are identical in 49 of 174 positions, a degree of homology equivalent to or greater than that found between human myoglobin and the α -chain of human haemoglobin¹¹. The most striking homologies occur in two major domains of the proteins: from amino acids 1–58, and from amino acids 119–159. Of particular interest is the similar distribution of cysteine residues. Eight of the 11 cysteine residues in polyoma virus can be aligned with eight of the 11 SV40 cysteine residues. Each protein contains two regions (120–125 and 148–153) in which there are cysteine clusters, Cys X Cys X X Cys, in highly conserved hydrophobic environments. Furthermore, it is remarkable that most cysteine residues are located in the carboxy terminal portion of the proteins. Only one cysteine residue occurs in the amino terminal region (position 38) of the SV40 protein.

In the case of SV40, T antigen is encoded by two discontinuous segments of the early region from ~0.65 to 0.60 map units and from 0.54 to 0.17 map units; t antigen is encoded by a continuous segment from ~0.65 to 0.55 map units; it shares a common amino terminal sequence with T antigen^{3,12,19} and, in addition, contains a unique sequence encoded by the region from 0.60 to 0.55 map units. The arrangement of the coding regions for polyoma virus T antigen and t antigen is presumably similar to that of SV40. One class of transformation-defective polyoma mutants, the *hr-t* mutants, map in the region coding for t antigen¹¹. They are defective in the expression of t antigen but they express a full size T antigen. It is interesting to compare the location of the presumed homologous regions with the location of coding regions for t antigen and T antigen and with that of known early functions. The major homology domain from 1 to 58 amino acids is located in the region common to T antigen and t antigen (0.65–0.60 map units). Of special interest is the fact that the second domain (amino acids 119–159) is located in the unique part of t antigen (0.60–0.55 map units), and is the region containing the highly conserved cysteine clusters. The latter region also codes at least for part of the polyoma hr-t function¹¹, and in the case of SV40 is required for transformation but not for lytic growth²⁰.

It was of interest whether t antigen and/or T antigen contains sequences that are homologous to any of the proteins whose amino acid sequences have been determined. One of the most conserved amino acid residues in any series of homologous proteins seems to be the cysteine residue. We, therefore, searched for sequences containing cysteine residues in arrangements similar to those found in the homologous regions of t antigen. A computer screening of approximately 800 protein and peptide sequences revealed that the sequence, Cys X Cys X X Cys, also occurs in the α and β subunits of all

members of the glycoprotein hormone family, including thyroid stimulating hormone, luteinising hormone, and chorionic gonadotrophin. Further resemblance between these proteins and the viral antigens is not immediately apparent, however. This array of cysteines was also found in two immunoglobulin γ -chains (of more than 100 examined) and in one kind of keratin. The latter is especially rich in cysteines and may be expected to have this sequence by chance. Finally, in Bowman-Birk soybean trypsin inhibitor, the sequence, Cys X X Cys X Cys (X)₂₁ Cys X X Cys X Cys, was found which closely resembles the sequence, Cys X Cys X X Cys (X)₂₁ Cys X Cys X X Cys, in SV40 t antigen and Cys X Cys X X Cys (X)₂₂ Cys X Cys X X Cys in polyoma t antigen. It remains to be seen whether this resemblance is merely fortuitous.

We estimate that the sequence presented here contains less than one error per 100 base pairs, particularly in the potential coding region starting at position 188. The sequence has been determined from continuous and overlapping regions of both strands, and the correctness of the large part of the sequence and the deduced amino acid product is supported by the marked homology with the SV40 t antigen, and the fact that only one reading frame is open throughout this region, the other two frames being closed by multiple termination codons. The potential translation products of regions of the other two closed frames show much less homology with the SV40 protein, further supporting the correctness of most of the sequence presented here. Formal amino acid sequence analysis will, of course, be required to confirm and/or correct the protein sequences deduced from the nucleotide sequences.

Previous hybridisation studies have suggested a common evolutionary origin of the papovaviruses SV40, polyoma, BK and JC^{6,7,13–15}. Only a small amount of homology between the late regions of the genomes of polyoma and SV40 has been detected⁶, but not in the early region. The kind of microhomology reported here for the early region can therefore be easily missed by the standard hybridisation techniques. The striking homologies in the nucleotide sequences of the early regions of polyoma virus and SV40 and in the deduced amino acid sequences, in particular the cysteine clusters, provide further strong evidence that these two viruses have a common evolutionary origin. Moreover, these data suggest that the tertiary structure of their t antigen and T antigen are very similar.

We thank Dr Sherman Weissman and Dr Walter Fiers for use of their SV40 nucleotide sequences before publication. T.F. thanks Dr Fred Sanger for his hospitality. This work was supported by grants CA 16115 and CA 15365 from the NIH and the Guggenheim Foundation.

THEODORE FRIEDMANN

Department of Pediatrics,
School of Medicine,
University of California, San Diego,
La Jolla, California 92093

RUSSELL F. DOOLITTLE

Department of Chemistry,
University of California, San Diego,
La Jolla, California 92093

GERNOT WALTER

Tumor Virology Laboratory,
The Salk Institute,
PO Box 1809,
San Diego, California 92112

Received 10 March; accepted 1 May 1978.

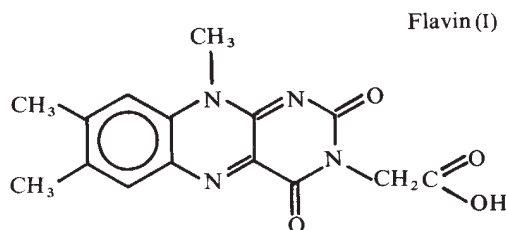
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Laser photo-CIDNP as a surface probe for proteins in solution

APPLICATIONS of chemically induced dynamic nuclear polarisation (CIDNP) have been limited so far to reactions of small organic molecules^{1,2}. We report here the observation of CIDNP in the 360-MHz NMR spectrum of a protein. The effect is generated by a cyclic reaction of a photo-excited dye with certain amino acid residues that are accessible to the dye. Of the common amino acids polarisation has been observed in tyrosine, histidine and tryptophan. The method distinguishes between exposed and buried groups and thus yields valuable information on the solution structure of proteins. Here we discuss the CIDNP observed in tyrosine residues of bovine pancreatic trypsin inhibitor (BPTI). The three-dimensional structure of BPTI (molecular weight 6,500) has been studied extensively by X-ray crystallography^{3,4} and by NMR^{5,6}.

We have described a difference method for the study of photo-CIDNP in biological macromolecules⁷. A solution containing the substrate (for example, a protein) and a dye in low concentration ($\sim 10^{-4}$ M) is irradiated by an argon ion laser (Spectra Physics, model 171) in the probe of the Bruker HX-360 NMR spectrometer operating at 360 MHz in the pulse Fourier transform mode. Gating of light and radiofrequency pulses is computer controlled. Alternating 'light' and 'dark' free induction decays are collected, which can be subtracted to yield the pure CIDNP spectrum. We have discussed elsewhere the CIDNP effects in a model reaction with *N*-acetyltyrosine, where 3-*N*-carboxymethyl lumiflavin (I) was used as a dye⁷.



The tyrosine showed strong emission for the 3 and 5 ring protons (*ortho* with respect to the phenolic OH group) and weak enhanced absorption for the 2,6 protons and the CH₂ group. The polarisation arises from a reversible hydrogen atom transfer from the phenolic OH of tyrosine (TyrH) to the photo-excited flavin (F). The flavin triplet is the reactive intermediate⁸. The reactions can be written as follows:



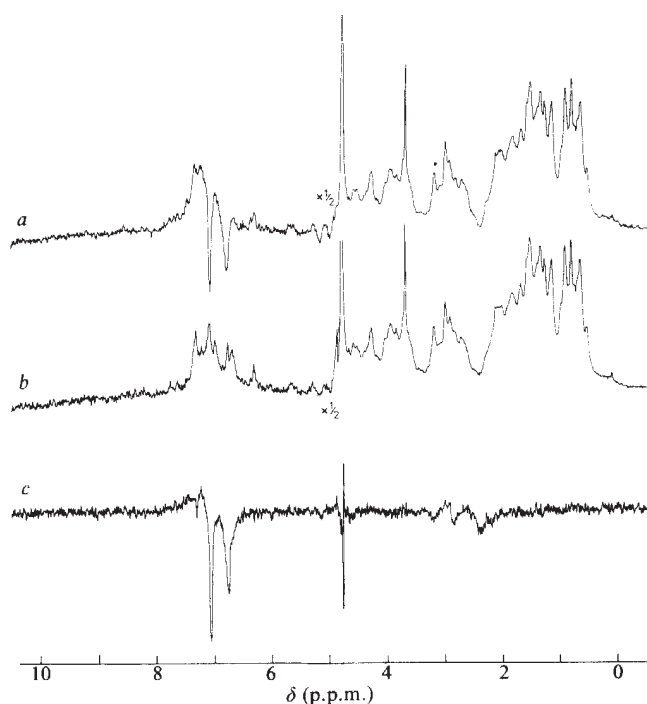
CIDNP (indicated by an asterisk) is generated in a spin-selective recombination of the radical pair, as in reaction (3). Its sign (emission or absorption) can be easily predicted from the spin-density distribution and g-factors of the intermediate radicals⁹. Reactions (1) to (3) constitute a cyclic process. Almost no net reaction occurs, although reaction (4) causes some bleaching of the dye.

The above procedure has been applied to BPTI, which has four tyrosines (Tyr 10, 21, 23 and 35) and four phenylalanines as the only aromatic residues. The backbone NH protons of BPTI (Sigma) had been pre-exchanged by heating in D₂O at 80 °C for 10 min and lyophilising. Figure 1 shows the 360-MHz ¹H NMR spectra obtained from a solution of 5×10^{-3} M BPTI and 2×10^{-4} M flavin (I) in D₂O at pH 5.6 (uncorrected meter reading). Figure 1a and b represent the light-irradiated and dark spectra, respectively.

The difference spectrum (Fig. 1c) clearly shows two emission lines at 7.04 p.p.m. and 6.74 p.p.m., which must belong to the 3,5 protons of two tyrosine residues. These lines have been assigned by Snyder *et al.*⁵ to Tyr 10 and Tyr 21, respectively. Inspection of the X-ray structure⁴ reveals that Tyr 10 and Tyr 21 lie at the surface of the protein, whereas Tyr 23 and Tyr 35 are buried in the interior. Hence, the photo-CIDNP result agrees with the crystal structure.

Figure 1c has some additional interesting features. The small emission line at 7.30 p.p.m. belongs to the 2,6 protons of Tyr 10. The polarisation is opposite to that found in *N*-acetyltyrosine (ref. 7) and arises from polarisation transfer¹⁰ from the 3,5 protons by dipolar cross-relaxation, counteracting the small

Fig. 1 360-MHz ¹H FT NMR spectra (40 pulses) of a D₂O solution of 5×10^{-3} M BPTI and 2×10^{-4} M flavin (I) at pH 5.6, temperature 21 °C. a, 'Light' spectrum obtained by irradiating the sample for 0.6 s by an argon laser (5 W, multiline) before data acquisition; b, 'dark' spectrum; c, photo-CIDNP difference spectrum, $a - b$. A 1-s broadband presaturation pulse was given before the light pulse. Light and dark free induction decays were taken alternately. Except for the light pulse the pulse sequence is the same for both spectra. The repetition time for one cycle (light + dark) was 15 s.



direct polarisation effect. Cross-relaxation is known to be important in proteins¹¹ and has also been demonstrated to occur in BPTI (B.D. Sykes, personal communication). For large molecules at high magnetic fields it gives rise to polarisation of the same sign as that of the primary polarisation. Most probably, it is also responsible for the emission lines in the crowded region around 3 p.p.m. (the positive components must belong to the β -CH₂ groups of Tyr 10 and Tyr 21). From titration studies¹² the ϵ -methylene lines of Lys 41 have been found to resonate at 2.96 p.p.m., close to where we find an emission (2.91 p.p.m.).

The proximity of Lys 41 and Tyr 10 has been inferred from the coupled titration behaviour of these residues¹². Thus, the emission line at 2.96 p.p.m. must probably be assigned to the ϵ -CH₂ group of Lys 41 polarised by polarisation transfer from Tyr 10. The emission lines at 2.35 and 3.20 p.p.m. may belong to other methylene groups of this residue. In small proteins like BPTI it is likely that polarisation is only transmitted to protons in the first shell around the primary polarised group. In larger proteins, where cross-relaxation is stronger, the effect may extend to larger regions in the protein.

Several other methods have been widely used to determine the extent of exposure of amino acid side chains. Therefore, it is interesting to examine some other properties of the tyrosine residues of BPTI in the light of their possible exposure to the medium. In Table 1 the *pK* values for tyrosine OH ionisation (determined by NMR⁶) are listed with chemical modification data for comparison with the present photo-CIDNP results and with the crystal structure. The *pK*s of Tyr 10 and Tyr 21 are close to that of aqueous tyrosine (*pK* 10.1), whereas those of Tyr 23 and Tyr 35 deviate considerably. This and the preferential nitration¹⁴ of Tyr 10 and Tyr 21 are as expected for freely accessible surface residues. Exhaustive iodination results in modification of Tyr 35 as well¹⁵.

Thus we have shown here that by using a dye as sensitiser photo-CIDNP can be generated in a native protein and that this may serve to distinguish between exposed and buried amino acid side chains. The method has some noteworthy features. First, being a NMR method it has the potentially very high resolution of this technique, which is capable of resolving individual groups of a protein in solution. In this respect it compares favourably with other spectroscopic methods. Second, signal enhancements of an order of magnitude are often observed. Last, by virtue of irradiation with visible light and a largely reversible photo-reaction, it is a mild method. In a review on buried and exposed groups in proteins Kronman and Robbins¹³ stated: "The ideal probe would be a small molecule or ion, which on interaction with a specific side chain, would send back a signal only if groups were freely accessible, but which would in no way perturb the conformation of the protein." We believe that the photo-CIDNP method comes close

to this ideal. Indeed, it is hard to envisage a more gentle perturbation than one involving nuclear spin state populations.

This work was carried out at the national NMR facility at the University of Groningen, supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO). We thank Spectra Physics for a loan of the argon laser, Dr F. Müller (Wageningen) for flavin, and Drs H. J. C. Berendsen and G. T. Robillard for stimulating discussions.

R. KAPTEIN
K. DIJKSTRA
K. NICOLAY

Department of Physical Chemistry,
University of Groningen,
Groningen, The Netherlands

Received 14 February; accepted 10 May 1978.

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Erratum

In the letter 'γ-Ray burst observed at balloon altitude' by J. Nishimura *et al.* *Nature* **272**, 337–338, in paragraph 6, line 3, $\delta = 44^{\circ}55' \pm 15'$ (not $44^{\circ}05'$).

Corrigendum

The attribution for the cover picture for *Nature* of 15 June (computer-generated space-filling model of insulin) should read R. J. Feldmann and T. K. Porter, of The Division of Computer Research and Technology, NIH.

Table 1 Some properties of the tyrosine residues of BPTI

Tyrosine	10	21	23	35
Chemical shift 3,5 protons (p.p.m.) (DSS)*	7.04	6.74	6.30	6.76, 6.80
<i>pK</i> *	9.9	10.4	11.5	11.1
Nitration†	3-NO ₂	3-NO ₂	—	—
Iodination‡	3-I	3-I	—	3,5-I ₂
Photo-CIDNP	E§	E§	—	—
X-ray structure	Exposed	Exposed	Buried	Buried

* From ref. 6, assignments to specific residues have been made in ref. 5.

† From ref. 14.

‡ From ref. 15.

§ Emission observed for 3,5 protons, present work.

|| From ref. 4.

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reviews

Social impact of technology

Eric Ashby

Technology and Social Shock. By Edward W. Lawless. Pp.616. (Rutgers University Press: New Brunswick, New Jersey, 1977.) \$6.95.

Do not be put off by the title of this book. The emotive words, hideously printed in garish colours on the cover, give the impression that this is another addition to the vulgar literature of doomsday prophecy. It is nothing of the sort. Indeed—to state my conclusion before I support it with evidence—this book will be indispensable to anyone who is reflecting seriously about technology assessment. Nothing quite like it has been published before.

In 1972 the Midwest Research Institute, with support from the National Science Foundation, embarked on a study of 45 case-histories of public concern over the secondary effects of some technologies. Public concern is generated by the mass media; therefore the source-material for this study is not scientific papers or government reports: it is the columns in daily or weekly journals which transmit, often in summaries full of distortion and inaccuracy, the complex and often confusing opinions of experts. In a pluralistic democracy public opinion carries great weight with politicians, so the legislative action which is taken to deal with hazards arising from technology is often more heavily influenced by what the mass media say about the hazard than by what the experts say about it.

Technology assessment, and public participation in decisions about such complex issues as whether to reprocess atomic material at Windscale and whether to sink coal mines in the Vale of Belvoir, are in a primitive stage of development. There are no well tested procedures, no reliable precedents, no criteria of reliability except the delayed verdict of history. It is urgently important that we look for patterns in this chain-reaction which begins when experts realise that an unexpected hazard has appeared, then becomes explosive when public opinion is aroused by the media, and ends only when action is taken to counteract the hazard (or when the apprehensions turn out to have been mistaken). The first stage in looking for patterns is to study case-histories

and to try to draw generalisations from them. That is what Edward Lawless has done in this book.

His selection of cases is arbitrary but it includes a very wide category of technological innovations which have had an unexpected backlash on society. Some are medical: the unanticipated secondary effects of oral contraceptives; the fluoridation controversy; the (apparently) unjustified scare about the drug dimethyl sulphoxide. Other cases are from the food industry: the scare, just before Americans cooked their turkeys for Thanksgiving in November 1959, that cranberries had been sprayed with 3-amino-1,2,4-triazole which, in large doses, produces tumours in mice; the cyclamate affair; the hazards of using diethylstilbestrol to fatten cattle. Another category of cases is about environmental pollution: the smog in Donora in 1948 which, like the London smog of 1952, precipitated clean air acts; the discovery (by a Canadian graduate student) that fish in Lake St Clair, on the Canadian-American border, were carrying up to ten times the legal limit of mercury; the story of the delayed-action effects of asbestos on health and its consequences in the present controversy over taconite mining in Minnesota; the three eruptions of concern about detergents (damage from foaming, a scare about damage from the inclusion of enzymes, and the fear that phosphates would cause eutrophication). There are cases, too, about major construction projects: the Truman reservoir in Missouri which was opposed partly because it put at risk a rare fish *Polyodon spathula*; the storage of radioactive wastes in salt mines in Kansas; the project, still undetermined, to build a giant underground transmitter to transmit messages to nuclear submarines.

This is just a sample of the cases discussed in the book. There are in addition brief synopses of some 50 or more other cases. What is so admirable is the way in which each case is presented. A diagram, with a time-scale as abscissa, displays the origin of the technology, its first use, the date of the first apprehensions about hazards in its use, the date when the media transmitted the apprehensions to the public, the dates of protests, hearings,

government interventions, and so on. There follows a background description of the issue, for which the author has gone back to the technical papers to check the accuracy of the accounts which appeared in the media; then come the "key events and roles" in the transmission of the material from the obscurity of scientific papers or the secrecy of governments or industry to the spotlights of the press and television. A section marked "disposition" records the outcome of each episode, and there is a final "comment" which is cool and objective. Each case-history has a list of references, including some to scientific sources, but mainly—as this is the whole purpose of the book—to the sources which would be read by the man-in-the-street. The book is written in a clear and clinical style and the conclusions drawn from the case-histories are impeccably undogmatic.

The author, having warned the reader that one cannot make generalisations from a sample of 45 non-randomly selected case-histories, does make some cautious comments. Some issues (he cites supersonic transport and the Alaska pipeline) attract concern only from an elitist public opinion. Popular public opinion is aroused when the issue touches health or food. He confirms what is well known: that there is no correspondence between the intensity of concern and the magnitude of the hazard; support for work on cancer-cures, for instance, a disease responsible for 17.3% of deaths in the USA in 1969, greatly exceeds support for work on the cure of cardiovascular diseases, which were responsible for 52.8% of deaths. The case-histories do include a melancholy record of lack of foresight. In nearly 40% of the cases there were early warnings of danger which were disregarded; this was so for oral contraceptives, the foaming caused by hard detergents, and the health hazards of asbestos and mercury. In more than half the cases the technology was applied commercially "with less than adequate responsibility by its users." In two thirds of the cases there was some government agency nominally in charge of the technology (and presumably of its secondary consequences). Nevertheless, Lawless is optimistic about the possible value of technology

assessment. To the question: "Could TA have helped?" he says yes, in many of the cases it could have avoided both the hazard and the 'social shock' produced by publicity about the hazard. The second of these is harder to achieve than the first, because once the issue gets into a storm of controversy the responsible authority is driven to make crisis-decisions (as were made when some American States banned phosphate from detergents in the belief that nitrilo-triacetate (NTA) would be a safe substitute, although NTA is a chelating agent which might bring heavy metals into solution, and is a suspect carcinogen.)

One of the many lessons to be learnt from this book is the essential part played by the mass media in the ignition of public opinion about hazards arising from technological developments. In the popular press sober truth has to be sacrificed to circulation, the reservations have to be stripped off the expert's statement. "On the whole", writes Lawless, "the news media have not done a particularly creditable job of reporting scientific and technological events to the public over the last thirty years. They tend to overdo the bizarre or the scare aspects at the beginning of a case and seldom follow through to summarise adequately the resolution of an issue". Yet the news media are the main channel of communication between the mass of the public and the people who are responsible for getting the facts right and making the decisions.

The author of this book would not wish his preliminary study to be taken as a sufficient basis for generalisations about the social impact of technology. But I venture to draw three provisional conclusions from *Technology and Social Shock*. Industrialists are tempted (though some resist the temptation) to put profits before the protection of society. (Lawless wryly reminds us that the motor manufacturers in Detroit continue to make cars with speedometers which register up to 120 mph "even as speed limits are being reduced to fifty five miles an hour across the country.") Government officials and politicians are tempted to play safe over hazards; witness the nonsensical consequences which sometimes follow the application of the Food, Drug, and Cosmetic Act Amendment of 1958—the notorious 'Delaney Clause'. Editors and science reporters are tempted (though some resist the temptation) to put sales above sobriety when they report hazard-stories. It is for scientists to make technology assessments, but if the assessments are to be of any practical value, industry, government, and the press will have to learn to make better use of the assessments than they do at present. And the assessments themselves need to be frequently revised. They can never be more than intelligent guesses and they will often be wrong, but, as this important book demonstrates, it is worthwhile to persist with them. □

Lord Ashby is a Fellow of Clare College, Cambridge, and Chancellor of the Queen's University, Belfast.

Evoked potential investigations

Progress in Clinical Neurophysiology. Vol. 1: Attention, Voluntary Contraction and Event-Related Cerebral Potentials. Edited by J. E. Desmedt. Pp.256. (S. Karger: Basel, 1978.) SFr./DM98; \$43.75.

THE title of this book, the first volume in a series based on a symposium held in Brussels in April 1974, will mislead those looking for papers on evoked potential correlates of attention in humans. This topic will be dealt with in volume 6. In the meantime, most of the papers in this volume demonstrate the ingenuity of both experimental design and data analysis required to further our knowledge of evoked potential correlates of behaviour.

The opening chapter contains important recommendations on publication criteria for evoked potential studies, and as such ought to have been published in an appropriate journal with a wider readership than this book will achieve. This chapter and the second, on evoked potential methodology in general, are relevant to a wider range of evoked potential investigations than are covered in the remainder of the book. The description of the methodology could have been oriented more towards the specific problems of recording the various slow potential shifts that are described in the remaining papers.

The main theme of the remaining papers concerns the scalp topography of slow potential shifts in man and their occurrence in identifiable brain mechanisms in animals. Important results include the differences in slow potential distribution during the sensory and motor phases of a task and the smaller hemispheric asymmetries seen for left-handers compared with right-handers in a variety of motor activities.

Geoff Barrett

Geoff Barrett is a Member of the MRC External Staff working at The National Hospital, London, UK.

Polymerisation chemistry

Polymerization Processes. Edited by C. E. Schildknecht and I. Skeist. Pp. 768. (Wiley: London and New York, 1978.) £27.

THIS volume is the latest of an authoritative and highly respected series of monographs in polymer science and is an updated and much revised version of the earlier *Polymer Processes* published in 1956.

The flavour and emphasis of the book is perhaps best illustrated by the fact that of the twenty-nine contributing authors only six are from academic institutions, the remaining twenty-three being associated with industrial organisations. The result is a book which gives an extensive and well balanced view of polymerisation chemistry with a very distinct and intentional bias towards the technological side of its subject.

Of the nineteen chapters, eight are directly concerned with step-reaction polymerisation and ten with addition polymerisation, the remaining chapter dealing with photochemically induced polymerisation and cross-linking reactions. The result is to provide a good account of the chemistry of all of the industrially important polymerisation processes coupled with a discussion of the way in which these processes are applied in commercial practice.

As an academic polymer scientist I found this book extremely interesting and stimulating. The account of basic chemistry is well done and comprehensive, and careful editing has ensured that a uniformly high standard is maintained. However, much of this material is available elsewhere and the most useful aspect of the book is undoubtedly the successful way in which the adaptation of chemistry to production scale processes is described. Most of the major commercial processes are covered, including both conventional melt, emulsion and suspension systems, and the more modern techniques such as bulk polymerisation of vinyl chloride.

This book should find ready acceptance among those who teach polymer science and wish to have a more practical account of their subject than that given in more conventional texts. It is uniformly well written, and although there are minor typographical errors the overall impression is of careful proof-reading and editing.

N. C. Billingham

N. C. Billingham is Lecturer in Chemistry at the University of Sussex, UK.

Picture-book of economic botany

The Power of Plants. By Brendan Lehane. Pp. 288. (John Murray: London, 1977.) £12.50.

A 'POWER OF' something or other, usually good, is a common Irish expression and this profusely illustrated book by an Irish author does indeed suggest a botanical Irish stew, thick, nourishing and well flavoured, but with far more varied vegetable ingredients. The manifold relationships of plants to man in feeding, healing, killing, pleasing, interesting and fooling him provide the main theme. As the author says at the beginning "Plants are the foundation of all existence of humans and animals, of society and civilization, of our dreams. They are the source of our nourishment and health, pleasures and ecstasies; they sustain religions, cultures, civilizations. In the end they can kill us, and return us to the soil on which they themselves feed".

The intent is to portray all this dependence graphically, in a vividly written easily understood text summarising a vast amount of information picked from a wide range of publications and in a wealth of coloured and black-and-white illustrations from an even wider range of sources. Such a medley raises the question of for whom the book is primarily intended. Although no professional botanist can fail to find something entertaining in the text or illustrations with which he has previously been unacquainted, it would seem fair to regard the book as an elementary introduction to economic botany and ethnobotany.

Covering so much, it necessarily does not go deeply into anything but succeeds in maintaining interest by surprising juxtapositions of statements and illustrations. Moreover, each of the five sections being chopped into numerous small more or less self-contained subsections, it makes little demand on the reader's mental digestion. It can be recommended to school teachers of biology as a source of unusual tidbits and to the general public as a revelation of the extraordinarily diversified and very important impact of the plant world on human life.

The first section, entitled "Power to Survive", deals with plants as part of the cycle of nature and with their successful adaptation to very different environmental conditions. The second, "Power to Sustain", covers the origins of agriculture and the major economic crops of the world, such as the bread-making cereals, rice, maize, potato and olive, together with vegetables, fruits

and culinary herbs; one illustration even shows 18 kinds of bread from the late Stone Age and Ancient Egyptian and Classical times onwards.

The third section, "Power to Heal and Kill", is concerned with medicinal and poisonous plants—the two are often the same, their effect for good or ill then depending on dosage—and a discussion of their lethal use provides the opportunity to portray such characters as Lucrezia Borgia, Catherine de Medici, Madame la Voisin, Crippen and Graham Young, as well as harmless and beneficial herbalists such as Dioscorides, Paracelsus, Bock, Mattioli, Fuchs, and their like. There is an excellent little chapter here on the "Art of the Herbals".

The fourth section, "Power to Alter Consciousness", has subsections on a diversity of hallucinogens and beverages, including peyote, tobacco, kava-kava, coffee, magic mushrooms, cola, tea, wine, beer, cannabis, morning glory, together with their erotic and magical associations. The fifth and last section, "Power on the Spirit", deals with plants in religion, art and horticulture.

The main attraction of this book obviously is in its 800 or more illustrations. These adorn every page and completely cover nearly half of them. Probably never before has such a diversity of illustrations relating to plants and to men associated with them been gathered into one book. They include reproductions from early manuscripts and printed herbals and from drawings and paintings of all kinds, as well as diagrams, modern aerial photographs and electron micrographs. To seek out such array must have cost an enormous amount of erudite enquiry

for many come from sometimes obscure sources. It is thus most regrettable and exasperating that these sources are rarely indicated with precision, sometimes not at all, or else inconsistently. Thus the illustration of *Aristolochia clematitis* on the title page has no caption at all; that of *Artemisia vulgaris* on p17 is said to be from "the seventh century Viennese edition of Dioscorides"; that of *Silene linifolia* (not *linoides*) on p161 from "the *Codex Aniciae Julianae*"; those of *Dipsacus*, *Urtica* and *Chenopodium* on pp164 and 165 from "the Viennese manuscript known as the *Wiener Dioskurides*". Thus one might assume that they come from at least four different works. In fact they are all reproduced from the excellent Graz facsimile of the celebrated sixth-century Byzantine herbal officially known as the *Codex Aniciae Iulianae Picturis illustratus nunc Vindobonensis Med. Gr. 1*, but often called *Codex Vindobonensis* of Dioscorides, brought to Vienna from Constantinople in the sixteenth century by the ambassador Busbecq and now in the Nationalbibliothek, Vienna. The illustration on p150 described as "A seventeenth-century woodcut" is a copy by P. Lambeck (Lambecius) of a coloured drawing in the same codex.

Nobody, however, should be put off from enjoying this attractive and unusual book by the few minor imperfections.

William T. Stearn

William T. Stearn, formerly a Senior Principal Scientific Officer in the Department of Botany, British Museum (Natural History), is a Visiting Professor in the Department of Botany and Agricultural Botany, University of Reading, UK.

Aspects of lichen ecology

Lichen Ecology. Edited by M. R. D. Seaward. Pp. 550. (Academic: London and New York, 1977.) £23; \$44.90.

THE edited volume has the particular advantage of being able to capitalise on available expertise in specific fields, but perhaps sometimes the disadvantage of being not well coordinated. If the latter applies it is either an editorial fault, or it reflects the embryonic or unbalanced state of research interest in the subject. The present volume falls into the latter category.

Most of the eleven chapters would seem to fall into three distinctive groups. Firstly, chapters 4 and 5 and parts of chapter 6 focus attention on the interactions of lichens with animals, a subject which has received increased attention recently—in particular, the association of distinctive invertebrate

populations with lichens. Further interest may be stimulated despite the difficulties of invertebrate identification being superimposed on those of lichens, an obvious case for cooperation between specialists. Chapters 3, 6 and 10 all touch on lichen-lichen interactions in relation to competition and successional phenomena (also neglected fields of study), and together these contributions provide a useful coverage of our present understanding of these 'ecosystem' topics.

As the editor states on p3 it is perhaps premature to attempt a worldwide review of lichens from the phytogeographical point of view. Nevertheless, having chosen to include accounts of three major biotic zones (chapters 6, 7 and 8), it is a pity that nothing is said of tropical and subtropical regions and that consideration of temperate regions is restricted to the British Isles. Admittedly these regions are floristically much richer and more

heterogeneous, but some review of the present state of knowledge would have been valuable.

Finally, lichenology in the British Isles is considered from various points of view in chapters 9, 10 and 11. The fact that chapter 10 could be written at all is an indication of the valuable work done by field lichenologists in the British Isles in recent years. It is unquestionably a most important paper and likely to become a classic, despite its preliminary nature. Those who have an aversion to the phytosociological approach will perhaps find the authors' early comments (pp296-304) helpful and encouraging. The reviewers only slight reservation concerns the proposed status to be accorded to man-made communities and seral stages of successions (p302).

Chapter 9 is the first substantial paper to be published on lichen conservation and is an excellent blend of the scientific approach and commonsense. Two points seem to be worth emphasising. The 'capture' of important lichen communities in nature reserves does not necessarily ensure their safety, even from reserve management, where this is likely to be more related to the needs of other plant or animal populations, for example, birds (p417). Secondly, the use of transplanting to introduce or reintroduce lichens into seemingly suitable areas perhaps needs to be monitored (p428).

One other important contribution, chapter 2, serves to emphasise the two major difficulties in lichen ecology, the problem of recognising the lichen species and that of defining the lichen

microhabitat.

A few specific minor criticisms might be made, for example, about the statements on mineral nutrition of saxicolous lichens: "by dissolving silicates (lichen) hyphae can... gain nutrients" (p33) and "An essential feature for colonization... is the ability to accumulate nutrients from rain or run-off" (p34). It remains to be seen what is the case. About pH: the presence/absence of lichens on leather, hair and wool is correlated with pH but not necessarily attributable to it directly (p257); pH 6.0 is not more acid than pH 5.8 (p274). Also, it is implied that lichenin and isolichenin are the only important cell wall components in lichens (pp89 and 130) and that the digestion of lichens is likely to require quite different enzymes from those needed for fungal digestion (p110). Neither of the assertions need be true. And finally, there is the confusing use of terms such as competitive/aggressive (pp55 and 205), vagant/erratic (pp19 and 230) and eutrophication/hypertrophication (p287). The otherwise useful Selected Glossary does not help overmuch with regard to their correct usage.

The book is, however, something of a collection of papers and might have been better entitled "Aspects of Lichen Ecology". It is nevertheless a most worthwhile and valuable venture and will be an essential reference for workers in the field of lichen ecology.

B. W. Ferry

B. W. Ferry is Lecturer in Botany at Bedford College, University of London, UK.

Replacing animals in biomedical research

Alternatives to Animal Experiments. By D. H. Smyth. Pp. 216. (Scolar Press: London, 1978.) Hardback £6; paperback £2.90.

SNATCHED from a life of obscurity and installed in contemporary plastic palaces, cultured cells, the most widely claimed alternative to animal experiments, are in danger of becoming Pygmalion's protégés [with apologies to Elsdale & Bard *J. Cell Biol.* 54, 626; 1972]. Whether cells lead primitive, less cultured lives when housed in more traditional residences is examined by Professor Smyth's book.

This book, written for the intelligent layman as well as the scientist, comprehensively surveys in an unemotional way the possibilities and limitations of cell culture as well as the many other methods suggested for replacing animals in biomedical research. As the

motives of the various anti-vivisection societies (like the aims of medical research) are diverse, Professor Smyth puts the issues in perspective by frequently quoting the feelings of the 158 experts consulted. In addition, as public esteem for medical research seems to be balanced by public concern for animal welfare, it seems likely that the demand for alternatives could involve the possible amendment of existing legislation. Accordingly the author provides information pertaining to this as well as to the activities of the anti-vivisection lobby in Parliament.

In summary Professor Smyth is to be congratulated as an animal lover and scientist for his objectivity in presenting the complex issues involved in the debate to date. This book can therefore be recommended to laymen, scientists and Parliamentarians alike who will hopefully use it as a source of reliable information in future debates on alternatives to animal experiments.

Alan J. Paine

Alan J. Paine is a member of the MRC Toxicology Unit, Carshalton, UK.

X-ray spectroscopy

X-Ray Spectroscopy. Edited by H. K. Herglotz and L. S. Birks. Pp. 513. (Marcel Dekker: New York, 1978.) \$49.50.

X-Ray Spectroscopy is a collection of articles by various authors, and concentrates on the technique as a tool for chemical analysis. As with most multiple author books, it suffers from lack of continuity of style and content and the typescript presentation makes the comprehension of equations very difficult. Its use to scientists working in more fundamental fields such as atomic physics, plasma physics or astronomy and space physics is virtually zero.

The book is divided into two sections with eight or nine chapters in each. The first section, "Methods and Instruments", contains two or three chapters which may be considered as an introduction to the subject. These are, on the whole, well written chapters, but the limitations on their length leave a number of loose ends so that anyone wishing to find a thorough grounding in the field would find them disappointing. In most cases these points are developed later in the book, but there is no logical way in which the topic can be followed. For example, in chapter one there is a brief mention of crystal rocking curves in association with wavelength dispersion techniques. Just how these curves are obtained is described briefly in the chapter on chemical analysis, but one would only find this out by accident. One chapter worthy of mention in this section is on electron probe microanalysers which, apart from an inadequate description of some of the equations used, is a useful introduction to the subject.

The second section of the book, "Applications", contains chapters on a wide variety of subjects from geology to museum objects. On the whole, these chapters are interesting and very readable, but I suspect that specialists in these particular fields would be better advised to save their money and find a relevant review article. The short chapter on "X-ray Astronomy and Other Exotic Applications" is so brief and superficial that one wonders why it was included at all.

One reason why many people may buy this book, however, is for the very large number of references that it contains, but I found the book itself rather disappointing.

T. A. Hall

T. A. Hall is Lecturer in Physics at the University of Essex, Colchester, UK.

Recent scientific and technical books

Physics

- HOFFMAN, E. J. *The Concept of Energy: Inquiry into Origins and Applications*. Pp.ix+573. ISBN-0-250-40153-3. (Ann Arbor, Mich.: Ann Arbor Science Publishers, Inc., 1977. Distributed in U.K. by John Wiley and Sons, Ltd., Chichester.) £18.60.
- KOMPA, K. L., and WALTHER, H. (edited by). *High-Power Lasers and Applications*. (Proceedings of the Fourth Colloquium on Electronic Transition Lasers in Munich, June 20-22, 1977.) (Springer Series on Optical Sciences, Vol. 9.) Pp.ix+228. ISBN-3-540-08641-2. (Berlin and New York: Springer-Verlag, 1978.) DM 48; \$24.
- LANDOLT-BORNSTEIN, *Numerical Data and Functional Relationships in Science and Technology* New Series. Editor in Chief: K. H. Hellwege. Group III: Crystal and Solid State Physics, Vol. 12: Supplement and Extension to Vol. 4. Magnetic and Other Properties of Oxides and Related Compounds. Part a: Garnets and Perovskites. By K. Enke *et al.* Editors: K. H. Hellwege and A. M. Hellwege. Pp.x+520. ISBN-3-540-08223-9. (Berlin and New York: Springer-Verlag, 1978.) DM 510; \$255.
- LIEBFRIED, G., and BREUER, N. *Point Defects in Metals 1: Introduction to the Theory*. (Springer Tracts in Modern Physics, Vol. 81.) Pp.xiv+342. ISBN-3-540-08375-8. (Berlin and New York: Springer-Verlag, 1978.) DM 89; \$44.50.
- MACKINNON, L. *Mechanics and Motion*. (Oxford Physics Series.) Pp.161. ISBN-0-19-851825-0. (Oxford: Clarendon Press; London: Oxford University Press, 1978.) Hardback £6 net; Paperback £3.25 net.
- PARIKH, Jitendra. *Group Symmetries in Nuclear Structure*. (Nuclear Physics Monographs.) Pp.ix+277. ISBN-0-306-31043-0. (New York and London: Plenum Press, 1978.) \$40.20.
- PINSKER, Z. G. *Dynamical Scattering of X Rays in Crystals*. (Springer Series in Solid-State Sciences, Vol. 3.) Pp.xii+511. ISBN-3-540-08564-5. (Berlin and New York: Springer-Verlag, 1978.) DM 86; \$43.
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- ELIAS, Hans-Georg. *New Commercial Polymers, 1969-1975*. Translated from the German by Mary M. Exner. Pp.xiv+211. ISBN-0-677-30950-3. (New York, London and Paris: Gordon and Breach, Science Publishers, 1977.) £13.
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Technology

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